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Studies on the pathogenesis of a canine abortion agent (Brucella canis) in dogs and other domestic animals

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STUDIES ON THE PATHOGENESIS OF A CANINE ABORTION AGENT (*BRUCELLA CANIS*) IN DOGS AND OTHER DOMESTIC ANIMALS

by

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INTRODUCTION

In 1966 a newly recognized contagious canine abortion syndrome was reported. The etiologic agent was regarded as a Brucella-like agent, but there was also speculation that it could be Bordetella spp. or Actinobacillus spp. Results of further studies on the causative organism indicated close similarity to Brucella and dissimilarity to bacteria belonging to other genera. There was considerable speculation that this newly recognized disease in dogs was similar or identical to brucellosis in other animals. The etiologic agent has become commonly known as Brucella canis.

The main features of the disease are reported to be generalized lymphadenitis, abortions, epididymitis, and prostatitis. Dogs are the only species in which naturally occurring disease has been reported. The disease has been diagnosed in at least 38 states since its recognition.

The clinical features of naturally occurring Br. canis infection in dogs have been described. There have also been descriptions of pathologic changes attributed to the infection. Some investigation of methods for diagnosis and control of the disease has been conducted. However, knowledge of the pathogenesis of the disease in dogs is still incomplete. There have been no direct comparisons of this disease with canine brucellosis caused by officially recognized Brucella species. Information about the pathogenicity of Br. canis for other

animal species, particularly domestic livestock, is only fragmentary.

This research was undertaken to investigate the comparability of Br. canis and Br. suis infections in dogs; to investigate the pathogenecity of Br. canis for cattle, swine, and sheep; and to provide further information on the pathogenesis of Br. canis infections in dogs and laboratory animals.

REVIEW OF LITERATURE

Brucella canis Infections

Carmichael (12, 14) was the first to report a new contagious reproductive disease in dogs, which occurred in New Jersey in 1966. Shortly thereafter, other reports of the same syndrome originated from different areas of the country (32, 59, 83). The main clinical feature of the disease was abortion during the last trimester of pregnancy without premonitory signs in Beagle bitches maintained in colonies. Information presented by Carmichael (13), Carmichael and Kenney (16), and Taul *et al.* (83) indicated that the disease was observed by dog breeders as early as 1962 and that the suspected etiologic agent had been isolated as early as 1965. During 1966-67 the disease was diagnosed serologically in over 800 dogs located in 38 states (16).

Initially, Carmichael (12) described his first isolate of the causative agent, made in June 1966, as a *Brucella*-like organism. Taul *et al.* (83) and Moore and Bennett (59) characterized the etiologic agent as a bacteria closely related to both *Bordetella* and *Brucella*.

Carmichael and Bruner (15) examined biochemical, growth, antigenic and morphologic characteristics of several strains of their canine abortion organisms and gas chromatographic signatures of culture extracts. In addition, they acknowledged the collaboration of other investigators who had had experience

with Brucella organisms. Characteristics in which the canine abortion organism differed from Bordetella bronchiseptica or Actinobacillus equuli (Shigella equirulis), but resembled Brucella suis or Brucella ovis (Ram Epididymitis Organism), and the resemblance of the canine disease to brucellosis in other species, were emphasized. They concluded that the dog organism was not identical with any presently recognized Brucella species and proposed the name Brucella canis.

Diaz et al. (26) compared the antigenic composition of the dog organism to Bordetella pertussis, Bordetella parapertussis, Bordetella bronchiseptica, Hemophilus influenzae, Pasteurella multocida, and Pasteurella pseudotuberculosis. Using agglutination tests, agglutinin absorption tests, and immunoelectrophoretic techniques, they concluded that the canine abortion organism was antigenically similar to rough Br. abortus, rough Br. melitensis, and to Br. ovis, but different from smooth Brucella and other organisms in the family Brucellaceae.

Jones et al. (40) reported that the dog organism had rough colonial morphology, resembled Br. suis in growth characteristics, had no demonstrable endotoxic activity, had lysozyme susceptibility similar to other brucellae, did not utilize erythritol, and had a cell wall structure similar to other Gram-negative organisms. They favored inclusion of the organism in the genus Brucella under the designation of Brucella canis.

Hoyer and McCullough (35) and McCullough (51) compared the polynucleotide sequences of the canine organism in DNA-DNA homology studies to those of recognized Brucella, Serratia marcescens, Bordetella bronchiseptica, Alcaligenes fecalis, Escherichia coli, and Francisella tularensis. The recognized Brucella species and the dog organism were indistinguishable in homology studies, whereas the other organisms studied had no detectable polynucleotide sequence homologies with Brucella.

Biochemical and oxidative metabolic characteristics of 10 strains of canine abortion organism were studied by Meyer (53). The different strains of canine organisms varied considerably in their ability to oxidize several substrates, including erythritol. Nevertheless, they were similar to Br. suis in metabolic characteristics and she recommended that the dog organisms be classified as Br. suis, biotype 5.

After isolation of the causative organism, Carmichael (12, 13) reproduced the disease experimentally by intravenous or subcutaneous inoculation and by oral, conjunctival, intravaginal, and contact exposure. He also stated that it could be transmitted to susceptible females by breeding to infected males. Others (58, 83) have also observed that the disease is rapidly disseminated among dogs closely kenneled, particularly at the time of breeding or when abortions occur. Moore (58) incriminated venereal transmission, congenital transmission, and also indicated that a high percentage of infected dogs may eliminate the organism in their urine.

The disease syndrome seems to be limited mainly to dogs of the Beagle breed that are maintained in large groups. This breed and management situation accounts for over 99% of the reported occurrences (16). However, a few cases have been diagnosed in other breeds, indicating that other breeds are susceptible. There is apparently no sex or age differences in susceptibility. Reports (64, 80) have intimated that dogs less than 6 months of age were not susceptible. However, Moore (58) reported the presence of bacteremia in newborn pups.

The salient clinical features of the disease in dogs are apparently limited. Most infected dogs have no visible signs of illness (13). Pyrexia has not been observed (58). Carmichael and Kenney (17) suggested that the failure of febrile response may be due to lack of endotoxin. Clinical pathology results have been inconsistent (58). Abortion without premonitory signs and a subsequent prolonged vaginal discharge, along with enlargement of palpable lymph nodes, are often the only signs noted in affected bitches (12, 13, 16, 32, 58, 83). Repeated abortions by a bitch are the rule, rather than the exception (16, 83). Many, if not all, infected females abort 2 or more times in succession (83). Up to 4 consecutive abortions have been reported (16). Aborted pups are often dead, but sometimes are still viable (16). In adult male dogs epididymal and prostatic enlargement, testicular atrophy, and dermatitis of the scrotum have been described (13, 16, 62). According to Moore and Gupta (60), none of the

clinical signs of Br. canis infection are pathognomonic because infection with beta hemolytic Streptococcus, Escherichia coli, and Herpesvirus canis, and various endocrine disorders may elicit similar signs.

Data presented by Pickerill (72) illustrate the economic impact of impaired reproductive performance associated with Br. canis infection. In a commercial kennel with approximately 600 breeding bitches the average number of pups weaned per bitch per year was 6.24 and 5.76 during the 2 years before Br. canis infection was diagnosed. Following infection, the average number was reduced to 1.51 pups.

After infection is established in dogs, bacteremia is a consistent finding (13, 16, 58, 86). Some dogs have remained bacteremic for over 2 years (58). Agglutinin titers also persist for $2\frac{1}{2}$ years or more in the majority of dogs that become infected. The magnitude and duration of agglutinin titers seem to be directly correlated to the persistence of bacteremia (16, 58). At necropsy or during the course of the disease, Br. canis has been isolated from fetuses, placentas, vaginal exudate, epididymides, prostate glands, testes, uteri, ovaries, mammary glands, milk, urine, lymph nodes, spleens, and livers of infected dogs (13, 16, 50, 58, 59, 62).

According to Moore (58), gross lesions at necropsy are not pathognomonic. Exudate in the uterine lumen, focal necrotic lesions in uterine mucosa, enlargement of lymph nodes and spleen, enlargement of epididymides and prostate glands, and

testicular atrophy have been described (13, 16, 58, 62). No characteristic gross lesions are found in pups; although the presence of serosanguinous abdominal fluid, subcutaneous edema, and subcutaneous hyperemia or hemorrhages are common findings (12, 16, 32).

Description of microscopic pathologic alterations have been limited mainly to changes observed in lymphoid organs and genital organs. Lymph node and spleen lesions have been characterized as a generalized diffuse lymphoid and reticular cell hyperplasia with accumulations of macrophages, lymphocytes, plasma cells, and neutrophils in the sinusoids (16, 58). The characteristic changes in genital organs have been interstitial lymphocytic infiltrations, ranging from focal perivascular accumulations to generalized infiltration with extension into and destruction of adjacent glandular parenchyma (16, 58, 62). In testes, degeneration of seminiferous tubules, fibrosis, and aspermatogenesis has been observed (62). Lymphocytic accumulations have also been observed in liver, lungs, kidneys, gallbladder, and urinary bladder (16, 17, 62). Necrotizing arteritis in target organs of gonadal steroids (prostate, scrotum, sheath, and vulva) has been described (17). Chronic meningitis and nonsuppurative encephalitis have been reported (17). The basic placental lesion is reported to be focal coagulation necrosis of chorionic villi and the presence of numerous bacteria within trophoblastic epithelial cells (16). Lesions of bronchopneumonia, myocarditis, renal

hemorrhages, lymphadenitis, and hepatitis were found in aborted fetuses (16).

Carmichael and Kenney (17) have postulated a diagrammatic scheme of the pathogenesis of the disease. Their postulate was based, in part, on representations of the pathogenesis of brucellosis in man. They theorized the following sequential steps: phagocytosis of Br. canis by leucocytes at the portal of entry, multiplication in regional lymph nodes, bacteremia, bacterial multiplication in other tissues, a complex immune response, and development of immunopathologic lesions, elimination of organisms, and immunity.

Some immunologic aspects of the disease have been investigated. Hypersensitivity reactions have been demonstrated in actively infected dogs with autolysates of whole Br. canis cells, but not with experimentally or commercially prepared hydrolysates (17). Dogs that recovered from the disease were resistant to reinoculation (17, 60). Spink (79) has reported the isolation of 19S agglutinins in pure form and at least 3 different 7S agglutinins.

Diagnosis of Br. canis infections has been based on isolation of the causative organism or serum agglutination tests, along with suggestive clinical signs. Procedures and tissues of choice for isolation of Br. canis have been described (13, 16, 59, 60, 61). Although no uniform standard procedure exists, there have also been descriptions of serum agglutination test procedures (13, 16, 61). Apparently, the

agglutination tests are fairly accurate in identifying dogs with existing or past infection (16, 62, 86), but nonspecific agglutination reactions occur with serums from dogs proved not to have been infected (72).

Attempts at therapy as a means of controlling the disease have failed (16, 58). Even though Br. canis has been shown to be sensitive to tetracyclines, sulfonamides, neomycin, streptomycin, novobiocin, and chloramphenicol in vitro (83), these therapeutic agents have been judged as only bacteriostatic in vivo (16, 58). Treatment results in a temporary cessation of bacteremia which returns when therapy is terminated. Nevertheless, it has been suggested that the practice of maintaining dogs on low levels of antibiotics might be associated with the marked diminution of the incidence of overt abortions in some kennels (17).

Results of preliminary attempts to develop immunization procedures have been reported (17). Brucella canis bacterins were unsuccessful except when 6 dogs were vaccinated with heat-killed organisms emulsified with Freund's incomplete adjuvant. Good immunity was afforded by a commercial killed Br. abortus 45/20 (a rough strain) adjuvant vaccine. However, both of the above preparations produced severe, extensive, and persistent swellings at the site of injection. A live variant of Br. canis, produced by laboratory manipulation, which was nonmucoid, of poor antigenicity, and of reduced virulence, was also used to vaccinate dogs. Vaccinated dogs were subsequently

protected against inoculation with virulent organisms, but the vaccine strain caused persistent infection and hyperplasia of lymphatic tissue.

Management factors are very important in controlling the disease, since the incidence of infection was much lower when dogs were caged individually (13, 58). Moore et al. (61) described a scheme that was used to successfully eliminate the disease from a large dog colony. Their procedure consisted of slaughtering dogs identified as infected by blood cultures. The blood culture results were supplemented with serum agglutination test results and dogs suspected of being infected were segregated.

Naturally acquired infections with Br. canis have been reported only in dogs. However, there have been 6 cases of human infection, mostly associated with laboratory accidents (82). Carmichael and Bruner (15) succeeded in producing infection in laboratory animals. They reported peritonitis, pleuritis, splenic enlargement, orchitis, epididymitis, and infection persisting up to 4 to 6 weeks postinoculation in mice, guinea pigs, and rabbits. Godzik (30) inoculated Br. canis into rabbits; and fetal abnormalities, metritis, and epididymitis resulted. He proposed that female rabbits would be a suitable model for studying Br. canis infections. One monkey (Macaca mulatta) has been experimentally infected by oral exposure (60). Cats are apparently moderately susceptible to Br. canis infection (72).

Pickerill (72) reported that nonpregnant swine, sheep, and cattle were highly resistant to infection by the oral-conjunctival route. None developed bacteremia, and organisms were not isolated from tissues taken at necropsy 4 to 6 weeks after inoculation. Also, 5 pregnant sheep inoculated orally, subcutaneously, and by combined oral and conjunctival instillation of approximately 1×10^9 organisms failed to become actively infected. No other details of methods or results were given.

Brucella suis and *Brucella abortus* Infections in Dogs

A comprehensive review of canine brucellosis caused by *Br. abortus*, *Br. suis*, and *Br. melitensis* was published by Morse (65). The majority of the available literature on the subject dealt with random serologic surveys and reports of spontaneous cases attributed to brucellosis. Many of the latter were lacking bacteriologic evidence of *Brucella* infection. Only a few controlled experiments have been reported. Dogs have been considered as potentially important in epizootiologic aspects of brucellosis, principally as mechanical vectors in dissemination of the disease. Dogs have been considered to be relatively resistant to natural infection with *Brucella spp*. In general, canine brucellosis has been characterized as a sporadic disease occurring in dogs associated with infected domestic livestock, as an asymptomatic disease, and as an infection rarely transmitted to other animals. Meyer (53) has contested the theory that the dog is a terminal host for

Brucella by citing several instances wherein transmission of brucellosis from dogs to man has been suspected.

The information presented by Morse (65) and Morse *et al.* (66, 67) indicates that dogs can be infected experimentally with *Br. suis* and *Br. abortus*. Infected dogs may harbor *Brucella* in lymph nodes for long periods after exposure. Development of agglutinins after exposure is common. Bacteremia has been demonstrated but is unusual. Females in estrus or pregnancy are apparently more susceptible to infection than females with a dormant genital tract, male dogs, or puppies. Abortions, chronic vaginal discharges, epididymitis, and orchitis do occur as a result of *Brucella* infection. Loss of weight, undulating fever, and lameness have also been attributed to canine brucellosis.

Wipf *et al.* (90) investigated the microscopic lesions due to *Br. abortus* infection in dogs, and also reviewed the fragmentary information available on pathologic aspects of canine brucellosis. They observed granulomata, fibrocytic proliferation, multinucleated cells, reticular scarring, necrosis, abscess formation, hemosiderosis, plasma cells, polymorphonuclear leucocytes, and eosinophils in lymph nodes from infected and control dogs. They concluded that the lesions were not specific, and were not necessarily related to the infection. They also described granulomatous lesions in the kidney and liver of a puppy born to an infected dam. There was no serologic or bacteriologic evidence of infection in the

puppy. Feldman *et al.* (29) attributed multiple focal purulent prostatitis, focal granulomatous hepatitis, and focal granulomatous pneumonia to experimental *Br. suis* infection in a male dog.

MATERIALS AND METHODS

Experimental Designs

Experiments with dogs

The canine abortion syndrome has frequently been compared to brucellosis in other animal species. Since there is little information on brucellosis in dogs caused by recognized species of Brucella, it appeared that a direct comparison in dogs would be a more valid approach. Because Brucella suis, type 3 appeared to be closely related to Br. canis in physiologic characteristics (53), a representative strain[1] of this biotype was chosen for the comparison. A subculture of Carmichael's original isolate[2] was used as the Br. canis strain.

The experiments were done in three stages. A preliminary experiment with 5 pregnant bitches (2 exposed to Br. canis, 2 exposed to Br. suis, and 1 nonexposed control) was conducted to determine some of the principal features of Brucella infection in dogs. Second, 7 pregnant bitches (3 exposed to Br. canis, 3 exposed to Br. suis, and 1 nonexposed control) were used in an experiment wherein the main objective was to determine pathologic changes in placentas. In the third

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[1] Brucella suis, type 3, strain 688; isolated in 1968 from swine of a herd in Iowa.

[2] Brucella canis, strain RM6-66 (American Type Culture Collection No. 23365); obtained from L. E. Carmichael, Cornell University, Ithaca, N.Y.

experiment 5 four-month-old pups were utilized (2 exposed to Br. canis, 2 exposed to Br. suis, and 1 nonexposed control) in an attempt to follow development of pathologic changes in lymph nodes.

The experimental dogs were Beagles obtained from 2 commercial suppliers, the 5 dogs for the first experiment from one kennel and the remaining dogs from another kennel. The ages of the pregnant bitches ranged from 1 to 7 years and the stage of gestation at the time of exposure to Brucella ranged from 10 to 37 days after breeding. The dogs originated from kennels with no history of reproductive problems and with no known Br. canis infection and were also serologically negative, using Br. abortus and Br. canis antigens for testing.

During the course of the experiments all dogs were maintained in isolation rooms with stable atmospheric conditions. They were fed a combination of dry dog ration,[1] evaporated milk, and a prescription diet,[2] mixed to provide high palatability as well as the recommended caloric intake. Dogs receiving different exposure material were kept in separate isolation rooms and those receiving the same exposure were kept in separate pens of the same room.

Exposures to Br. canis or Br. suis were performed by dropping 0.05 ml. of suspensions containing approximately

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[1] Wayne Dog Food - Allied Mills, Inc., Chicago, Ill.

[2] p/d - Institutional Products, Topeka, Kansas.

5×10^7 viable organisms/ml. into the conjunctival sac of each eye; therefore, the exposure dose was approximately 5×10^6 organisms/dog. After instillation of infectious material, the eyes were held closed for 2 to 3 minutes to reduce external loss of exposure material. Each group of dogs, after the first experiment, was exposed to cultures isolated at necropsy of the previous group of dogs.

After exposure all dogs were observed twice daily for signs of illness. Rectal temperatures were determined at that time. Blood samples (10 to 20 ml./sample) were collected from the jugular vein of each dog at semiweekly intervals and examined bacteriologically and serologically. The posterior vagina of each bitch was also examined bacteriologically at semiweekly intervals by use of a swab technique.

In the third experiment one lymph node from each dog was removed surgically at monthly intervals after exposure. Either superficial cervical (prescapular) or popliteal nodes were removed in the following order: 1st month - left superficial cervical; 2nd month - right superficial cervical; 3rd month - left popliteal. For surgery the dogs were given a tranquilizer[1] as preanesthetic, then anesthetized with thiamylal sodium.[2] The lymph nodes were removed aseptically, weighed,

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[1]Acepromazine maleate, Ayerst Labs, Inc., New York, N.Y.

[2]Surital sodium (2.5%), Parke, Davis & Co., Detroit, Mich.

and sectioned. A portion of each was placed in fixative for histologic examination and the remainder was used for bacteriologic examination.

The time of necropsy was predetermined at the beginning of each experiment. In the first experiment the day of necropsy was planned to be the third day postpartum or postabortion. In the second experiment the dogs were necropsied 7 weeks after their breeding dates. Necropsy of pups in the third experiment was planned for 5 weeks following the cessation of detectable bacteremia.

The dogs were killed by intravenous injection of 3 ml. of 10% succinylcholine chloride (77). Fetuses *in utero* were apparently not affected by the succinylcholine and it was possible, if examined within 5 minutes of death of the dam, to estimate the viability of the fetuses according to their appearance, movements, and response to touch.

All pups or fetuses were weighed and measured at the time of necropsy. In addition, all lymph nodes collected were weighed for comparison with the body weight of each dog as one measurement of lymph node enlargement.

Gross lesions were observed and recorded and sections from the following tissues of each dog were placed in fixative for later histologic examination: placentas, uterus, ovaries, mammary glands, spleen, liver, lungs, tonsil, kidneys, adrenal glands, and suprapharyngeal, superficial cervical, bronchial, internal iliac, and superficial inguinal lymph nodes. In

addition, sections of liver, spleen, lung, kidney, and thymus from each puppy or fetus were fixed for histologic examination.

Samples of each of the above tissues were also examined bacteriologically in addition to samples from the: vagina, cervix, amniotic fluids, allantoic fluids, milk, bone marrow from both femora and both radii, bile, trachea, urine, brain, and cerebrospinal fluid from each dog and stomach contents and heart blood from fetuses or puppies. Also, the mandibular, parotid, axillary, sternal, hepatic, ileocecal, and popliteal lymph nodes were collected for bacteriologic examination along with all remaining portions of the suprapharyngeal, superficial cervical, bronchial, internal iliac, and superficial inguinal nodes.

Experiments with cattle, swine, and sheep

Cattle Six pregnant Holstein heifers were obtained from the NADL herd. They had been isolated from other livestock for approximately 6 months prior to initiation of the experiment. During the course of the experiment they were housed in large animal isolation rooms and fed a complete, pelleted ration at maintenance levels. These heifers were exposed at 117 to 140 days of gestation to a culture from the second canine passage of Br. canis, strain RM6-66. The exposure was approximately 1 X 10^8 viable organisms by the conjunctival route. This dose represented about 100 times the

exposure required to produce infection in 90% of susceptible cattle with virulent Br. abortus, using similar methods (47).

After exposure, the heifers were observed daily for clinical signs of infection. Blood samples for bacteriologic and serologic examination were collected at semiweekly intervals for the first 4 weeks postexposure, weekly for the next 4 weeks, and biweekly thereafter. On the day of parturition uterine fluids, placentas, milk and blood from the heifers and rectal contents and blood from their calves were collected and examined bacteriologically. Whey from colostral milk of the dams and serum from the newborn calves were also tested for Br. canis antibodies.

One heifer was killed and necropsied at 4 weeks postexposure; the remaining heifers were necropsied at 2 or 3 days postpartum. They were killed by intramuscular injection of 5 ml. of 10% succinylcholine chloride. Tissues collected at necropsy for bacteriologic examination included spleen, liver, kidneys, adrenal glands, lungs, tonsils, cerebrospinal fluid, bile, femoral bone marrow, joint fluids from the humeroradial and femorotibial articulations, ovaries, uterus, uterine contents, cervix, vagina, mammary glands, and the mandibular, suprapharyngeal, parotid, atlantal, prescapsular, posterior mediastinal, bronchial, renal, coeliac, hepatic, mesenteric, internal iliac, deep inguinal, prefemoral, popliteal, and supramammary lymph nodes. Tissues were to be collected for

histologic examination only if gross lesions were apparent in them.

Swine The swine were crossbred boars and gilts (6 of each sex). They were hysterectomy-derived and were isolated from other livestock during their entire life. Maintenance of the swine and the general design of the experiment were similar to that described for cattle.

The gilts were exposed to Br. canis at 41 to 51 days of gestation when they were 10 months of age. The boars were 9 months old at exposure. All the swine were exposed by the conjunctival route to 1 X 10^{10} viable organisms of a second canine passage culture of Br. canis, strain RM6-66. This exposure dose was about 400 times that required to produce infection with Br. suis in 80% of swine, under similar conditions. After exposure, blood samples were collected semiweekly for the first 4 weeks and weekly thereafter. At parturition, uterine fluids, placenta, milk, and blood from each gilt were collected and examined bacteriologically, as were the liver, lungs, spleen, stomach contents, and blood of at least 3 newborn pigs from each litter.

The boars were killed (5 ml. of succinylcholine i.v.) and necropsy was performed at 4, 6, and 8 weeks postexposure, 2 boars at each time period. The gilts were killed and necropsied at 71 to 94 days postexposure, which ranged from 4 to 27 days postpartum. Tissues collected for bacteriologic examination were: liver, spleen, lungs, kidneys, tonsils,

adrenal glands, joint fluids from 3 articulations of each leg, bone marrow from the femora and humeri, all parts of reproductive tracts, urine, cerebrospinal fluid, and the mandibular, suprapharyngeal, parotid, middle cervical, sternal, bronchial, mediastinal, gastrohepatic, renal, splenic, mesenteric, internal iliac, superficial inguinal, prescapular, prefemoral, and popliteal lymph nodes.

Sheep The design of this experiment was very similar to those described for cattle or swine and only the exceptions are mentioned.

Six yearling Columbia rams, 4 yearling Columbia ewes, and 2 four-year-old, virgin Suffolk ewes from the NADL flock were utilized in the experiment. The ewes were exposed to Br. canis 29 to 60 days after breeding. The exposure dose for each sheep was 1 X 10^{10} viable organisms, a quantity 10 times that shown to produce a high infection rate with Br. ovis, using similar methods (69). Blood sample collections were done at the same intervals as described for swine. Semen samples were also collected from rams, by electroejaculation, at biweekly intervals and used for bacteriologic examination and semen evaluations. The rams were necropsied at 4, 8, and 12 weeks postexposure (2 at each time). The ewes were necropsied at 3 days postpartum or 93 to 108 days postexposure. Lambs were necropsied the same day as their dams; and liver, spleen, kidneys, lungs, blood, stomach contents, and suprapharyngeal lymph nodes of each were examined bacteriologically. Otherwise,

the samples collected at parturition and necropsy were the same as those described for cattle.

Experiments with guinea pigs

Formal and informal reports that Br. canis caused pneumonia, pleuritis, peritonitis, splenitis, orchitis, epididymitis, and lymphadenitis in guinea pigs had indicated that this might be a useful small laboratory animal for study of Br. canis infections. Consequently, an experiment on the pathogenesis of Br. canis and Br. suis infections in guinea pigs was conducted.

Thirty male and 30 female guinea pigs, weighing between 425 and 550 Gm. each, were obtained from the SPF guinea pig colony at NADL. Twelve of each sex were inoculated with Br. canis, strain RM6-66; the same number with Br. suis, type 3, strain 688; and 6 of each sex were not inoculated and served as controls. The inoculations consisted of intraperitoneal injection of 1 ml. of suspensions containing approximately 1×10^6 viable organisms/ml.

Ten guinea pigs each were killed at 3, 7, 10, 14, 21, and 28 days postinoculation. These groups of 10 guinea pigs each included 2 males and 2 females inoculated with Br. canis, 2 males and 2 females inoculated with Br. suis, and 1 male and 1 female control. At necropsy blood samples were collected for serologic testing, gross lesions were observed, spleen weights and body weights were determined, and tissues (spleen, liver,

lungs, testes or uterus, and prefemoral lymph nodes) were collected for bacteriologic and histologic examination.

Subsequent to the above experiment, the virulence of Br. canis was tested in guinea pigs from the standard NADL colony using a different experimental design. In this experiment, 20 male, 400 to 500 Gm. guinea pigs were inoculated subcutaneously with approximately 3×10^6 viable organisms. Half of the group were killed and necropsied at 3 weeks postinoculation and the other half at 6 weeks postinoculation. The necropsy included determination of spleen weights and body weights, observation of gross lesions, serologic testing of blood, and bacteriologic examination of spleens. For comparative purposes, Br. suis, type 3, strain 688; Br. suis, type 1, strain 3b (a high virulence strain); and Br. abortus, strain 19 (a low virulence strain) were tested for virulence in guinea pigs, using the same methods at the same time. A group of 20 uninoculated control guinea pigs was also utilized.

A numerical evaluation of the severity of disease in each guinea pig was made. This index of infection provided a maximum of: 8 points for isolation of Brucella, 7 points for gross pathologic changes, 3 points for serologic response, and 2 points for clinical response, making a total of 20 points possible for each guinea pig. The number of points assigned for each parameter was dependent on the relative number of organisms isolated, severity and extent of gross lesions, and the degree of serologic and clinical responses. For comparison

of Brucella strains, the total numerical assignment for each group of guinea pigs was calculated.

Experiments with macrophage cultures

As a corollary to the above experiments, the behavior of normal guinea pig peritoneal macrophages toward Br. canis, strain RM6-66 and Br. suis, strain 688 was determined. The methods used were guided by those described for similar experiments (33, 34, 43, 73).

Ten normal guinea pigs were injected intraperitoneally with 10 ml. of 0.15 M NaCl 24 hours before collection of macrophages. The guinea pigs were killed with thioamylal sodium and the peritoneal cavities were washed with 10 to 20 ml. of Hanks medium[1] containing heparin (5 units/ml.). The cell suspensions were collected and processed in sterile siliconized tubes. The cells were washed twice in Earles medium;[2] then suspended, counted, and diluted in Earles medium containing 30% normal guinea pig serum to a concentration of 1×10^6 cells/ml. One milliliter of this suspension was put in each of the Leighton tubes needed for the experiment and incubated for 2 hours to allow attachment of the cells. The supernatant fluid was

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[1]Hanks medium (22) also contained 0.5% lactalbumin hydrolysate.

[2]Earles medium (22) also contained 0.5% lactalbumin hydrolysate, 1% SPF calf serum, penicillin (100 units/ml.), dihydrostreptomycin (0.1 mg./ml.), Kanomycin (0.1 mg./ml.), and Fungizone (2 µg./ml.).

decanted, contents of the tubes rinsed with Hanks medium, and 1 ml. of Hanks medium containing 30% normal guinea pig serum with or without Brucella (1 X 10^7 viable organisms/ml.) was added. The tubes were placed in a 37 C. incubator and, after 2 hours incubation, one third of the tubes were removed for analysis. The fluids in the remaining tubes were removed, the contents rinsed with Hanks medium, and 1 ml. of Hanks medium containing 30% normal guinea pig serum and 10 µg./ml. dihydrostreptomycin was added. The latter cell cultures were analyzed at 21 and 44 hours (one half at each time) after addition of Brucella.

The analyses included: viability counts of intracellular Brucella; staining of cells on flying coverslips for microscopic estimation of numbers of intracellular Brucella and proportion of macrophages in the cell population; and determination of cytotoxic effects. To prepare material for viability counts, the medium was removed from the tubes, the contents were rinsed with Hanks medium, and fresh Hanks medium was added. The contents of the tubes were frozen and thawed to rupture the macrophages, and the number of colony forming units of Brucella in the resulting material was determined by a plate counting technique (1). The flying coverslips in Leighton tubes for microscopic evaluation were rinsed with 0.15 M NaCl solution and treated with methanol (5 minutes) and 10% formalin (10 minutes) for fixation of cells. They were stained with Giemsa stain, Taylor's method (84), or immunofluorescent

staining and examined microscopically. Cytotoxicity was estimated using the Trypan Blue method described by Kessel and Braun (43).

The experiment was designed so that one third of the cell culture tubes were inoculated with Br. canis, one third with Br. suis, and one third remained as controls. One third of the tubes were analyzed at each time period (2, 21, and 44 hours). Duplicate tubes were set up for each of the 5 described analytical procedures for each time period and for each inoculum (Br. canis, Br. suis, and controls). The experiment was repeated once using the same methods.

Bacteriologic Procedures

The mediums used for growth and maintenance of bacterial cultures at the beginning of the experiments were tryptose agar containing 5% bovine serum (TBS) (70) and potato infusion agar (3). Although potato infusion agar is one of the most suitable mediums for maintenance of Brucella cultures, it did not support the growth of Br. canis adequately. Therefore, only TBS medium was used routinely for growth and isolation of bacteria thereafter. A selective medium, TBS containing antibiotics (TBSA) (70), was used in bacteriologic examination of tissues and fluids when the presence of bacteria other than Brucella was anticipated. In addition, a modified MacConkey agar (91) was used in bacteriologic examination of respiratory tracts from dogs, swine, and guinea pigs at necropsy.

All cultures used in this research were maintained as lyophilized cultures because the viability of Br. canis decreased rapidly when stored on agar or broth medium at 4 C., 25 C., or 37 C. Cultures, to be used for any purpose, were not carried on TBS more than 2 passages from the lyophilized state or isolation plates (TBS in petri dishes). Mass transfer of cultures was the routine procedure; selection of colonies was not done.

All cultures for tissue culture and guinea pig inoculations, animal exposures, and tests of characteristics were propagated on TBS slants at 37 C. for 40 to 48 hours and washed off the agar surface with 0.15 M NaCl. Bacterial concentrations were estimated by density measurements[1] and consultation of previously determined density vs. viable cell count data and appropriate dilutions in 0.15 M NaCl were made. In the case of inoculation or exposure cultures, the actual viability count of each suspension was determined (1).

Morphologic, physiologic, and serologic characteristics of numerous Brucella strains in the NADL Brucellosis Research culture collection, at least one isolate from each infected animal, all exposure cultures, and cultures of other bacteria used for serologic comparisons were determined. The tests included those recommended for general classification of

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[1]Spectronic 20 Colorimeter, Bausch and Lomb Optical Co., Rochester, N.Y. or Model 7 Photo-Nephelometer, Coleman Instruments, Inc., Maywood, Ill.

bacteria (10, 21), conventional tests for speciation of Brucella (1), and tests for dissociation (11, 31, 89). These tests were necessary to develop familiarity with the discrete characteristics of the canine abortion organism and its relationship to similar agents.

Bacteriologic examination of most tissues and fluids collected during the course of animal experiments were conducted by described methods (25). Usually, the bacteriologic examinations at necropsy required 100 to 150 plates of medium for each bovine, porcine, ovine, or canine. In all attempts to recover Brucella, the inoculated medium was incubated for 5 to 7 days at 37 C. before discarding. Unless the precise concentration of organisms in tissues was determined, the concentration was estimated by rating them according to the number of colonies arising on the medium of each plate.

The number of colony forming units per gram (CFU/Gm.) of tissue was determined in lymph nodes from dogs and spleens from guinea pigs. This was accomplished by weighing the tissues, grinding[1] them in a measured quantity of 0.15 M NaCl, and determining the number of organisms in the resulting slurry with the plate counting technique. The lymph nodes from each dog, except one, were pooled before grinding.

Blood samples for bacteriologic examination (50 to 60 ml. from cattle, 30 to 40 ml. from swine and sheep, and 5 to 10 ml.

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[1]Model 702B Waring Blendor, Waring Products Corp., Winsted, Conn., operated at 8,000 to 10,000 rpm for 1 minute.

from dogs) were collected aseptically by venipuncture. Each sample was placed in a sterile container with a similar quantity of trypticase-soy broth containing 1% sodium citrate. The blood-broth mixtures (hemocultures) were placed in a freezer at -70 C. for 1 to 2 hours, thawed at 37 C., and 0.25 ml. quantities of each sample were spread over the surface of the medium in 2 to 4 plates. The hemocultures were also incubated at 37 C. and subcultured on TBS and TBSA mediums after 2, 7, and 14 days incubation.

Serologic Procedures

Development of serologic test methods for detecting Br. canis antibodies was necessary prior to initiation of animal experiments. These tests were needed to aid in selection of animals and monitoring the course of infections produced. It was known that Br. canis antiserum would not react with smooth Brucella significantly; therefore, standard Brucella antigens would not serve the purpose. Furthermore, the mucoid and auto-agglutinating properties of Br. canis required modified procedures.

In the procedure finally developed, Br. canis, RM6-66 was propagated on TBS + 1% additional agar medium in Roux flasks for 24 hours at 37 C. The bacterial growth was harvested in 0.15 M NaCl and the resulting suspensions were heated at 60 C. for 1 hour to kill the Brucella. The antigen was washed once and resuspended to the same density as concentrated Br. abortus,

1119-3 tube agglutination test antigen (4), and diluted similarly for use in agglutination tests. A buffered saline[1] diluent that proved to be satisfactory for keeping the antigen in suspension was used in the agglutination test procedures and in antigen preparation. The tests were incubated for 40 to 48 hours at 37 C. before reading and otherwise conducted according to prescribed procedures (4). The above procedure was considered preferable to reported methods (13, 16, 50, 61) after comparison with them.

A mercaptoethanol test (2) was frequently used along with the agglutination test in an attempt to distinguish types of agglutinins detected. This test was exactly the same as the agglutination test procedure, except that a 0.1 M 2-mercaptoethanol solution was used as the serum diluent, so that the final concentration of 2-mercaptoethanol in the tests was 0.05 M.

A complement fixation test, patterned after that described by Jones et al. (39), was used. A 3-ml. total volume was used in the test, and fixation was conducted at 37 C. for 1 hour. Sheep serums to be tested were inactivated at 60 C., other serums at 56 C. Since whole cell Br. canis antigen was found to be highly anticomplementary, a soluble antigen, similar to that used for diagnosis of Br. ovis infections (8), was

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[1]Buffered saline = 50 ml. 1 M KH_2PO_4, 50 ml. 1 M NaOH, 0.1 Gm. thimerosal, 1 Gm. gelatin, 900 ml. 0.15 M NaCl; pH 8.1 to 8.3.

prepared. Stock Br. canis, Br. abortus, and Br. ovis antigens were autoclaved at 121 C. for 15 minutes and centrifuged. The supernatant fluids served as the antigen for complement fixation tests.

An agar gel double-diffusion procedure, using agar medium[1] in petri dishes with wells 4 mm. in diameter and 12 mm. apart, was used for detection of serum precipitins. The soluble Br. canis antigen described, Br. abortus, 1119 antigen prepared in the same manner, and a sonicated Br. ovis antigen (7)[2] were used.

All canine serums collected during the experiments were tested for Br. canis antibodies with the tube agglutination (TA), mercaptoethanol (ME), complement fixation (CF), and gel diffusion precipitation (GDP) tests, for Br. abortus antibodies with the same test procedures, and for Br. ovis precipitins. All cattle, swine, and sheep serums were tested with the Br. canis TA, ME, CF, and GDP tests and with the standard agglutination tests for brucellosis (4). In addition, sheep serums were tested with Br. ovis CF and GDP tests and cattle serums were tested with the Br. ovis GDP test. Guinea pig serums were tested with the TA and ME procedures, using Br. canis and Br. abortus antigens.

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[1]Agar medium = 0.7 Gm. Ionagar, 0.3 Gm. NaH_2PO_4, 0.06 Gm. Na_2HPO_4, and 0.01 Gm. thimersol in 100 ml. distilled water.

[2]Obtained from G. M. Brown, NADL, Ames, Iowa.

For analysis of data obtained with the TA, ME, and CF tests, the greatest serum dilution in which 50% agglutination or fixation occurred was considered the end-point. Intermediate reactions were assigned numerical values by a method similar to that suggested by Morgan (63). Precipitation test results were classified as either positive or negative.

Since some other organisms belonging to the family Brucellaceae had been reported to cross react serologically with Br. canis (15, 26, 83), a serologic comparison was conducted. Antigens and antiserums were prepared from: Br. abortus, strain 19; Br. suis, strain 1330; Br. melitensis, strain 16M; Br. neotomae, strain 649; Br. ovis (Ram Epididymitis Organism), strain 692; Br. suis, strain PR-1;[1] Br. abortus, strain 45/20; Alcaligenes marshallii;[2] Moraxella bovis, strain EP-300;[3] Bordetella bronchiseptica, strain 546;[4] Bord. bronchiseptica, strain 711;[5] Pasteurella multocida, strain 1059;[6] and Actinobacillus equuli (Shigella equirulis).[7]

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[1]A rough mutant of Br. suis, type 1, strain 3b produced by laboratory manipulation.

[2]Recovered from cow's milk.

[3]Obtained from G. W. Pugh, NADL, Ames, Iowa.

[4]Obtained from D. L. Harris, VMRI, Iowa State University, Ames, Iowa.

[5]Recovered from guinea pig lung.

[6]Obtained from K. L. Heddleston, NADL, Ames, Iowa.

[7]Obtained from D. W. Bruner, Cornell University, Ithaca, N.Y.

Antigens for the comparisons were prepared in the same manner as described for Br. canis antigens. Antiserums against each strain of bacteria were produced in adult New Zealand White rabbits by single injections of live cultures of the smooth Brucella, weekly injections of live cultures of the Alcaligenes and nonsmooth Brucella (Br. canis, Br. ovis, Br. abortus 45/20, and Br. suis PR-1), and weekly injections of heat-killed (60 C. for 1 hour) cultures of the other organisms. Approximately 1 X 10^9 colony forming units of live cultures were injected each time and 50 X 10^9 organisms of killed cultures were given at each injection. All strains were injected intravenously, except for the Bordetella and Actinobacillus, which were injected intradermally, subcutaneously, and intramuscularly. The antiserums were derived from blood collected at 3 weeks after the initial injection.

All the antiserums were tested with each antigen using the TA, CF, and GDP procedures. Both soluble and whole cell antigens were used in CF tests. However, whole cell antigens of all nonsmooth Brucella were strongly anticomplementary; consequently, only CF tests using soluble antigen could be interpreted when nonsmooth Brucella strains were used as antigen. Results of the tests were categorized as: a strong reaction when the titer obtained with a heterologous antigen or antiserum was within 3 two-fold dilutions of the homologous titer, a weak reaction when the heterologous titer was within 4 to 6 two-fold dilutions of the homologous titer, and an

insignificant reaction when the titer difference was greater than 6 two-fold dilutions.

As a supplement to ME tests conducted on serums from animals exposed to Br. canis, 8 serums (2 each from cattle, swine, sheep, and dogs) were subjected to sucrose density gradient analysis, using a procedure similar to that employed by Rose and Roepke (75). One-half milliliter of 50% sucrose (W/V in 0.15 M NaCl) was placed in the bottom of each centrifuge tube, and a continuous gradient from 39% to 14% sucrose was formed with a Spinco gradient former. Antiserum (usually 0.5 ml.) was placed at the top of the gradient, and the material was centrifuged at 41,000 rpm for 18 hours in a Spinco Model LHV-2 refrigerated centrifuge with an SW 41 rotor. After centrifugation, 0.5 ml. fractions were removed with a micropipette and the agglutinin content of each fraction was determined with TA tests.

At least one sample of milk whey collected from each heifer and ewe after parturition was examined serologically. Whey was prepared by adding lactic acid (85%) to aliquots of milk until coagulation became evident (at pH 3 to 4). The sample was refrigerated overnight, centrifuged, and the pH of the supernatant material (whey) was restored to that of the original milk with 5 M NaOH. The wheys were tested for Br. canis agglutinins with the TA and ME tests.

Protein analyses were conducted on serums collected at weekly intervals from all experimental dogs to determine

whether the concentrations of serum protein components were altered as a result of Brucella infection. The analyses included total serum protein determination by the biuret method (6) and electrophoretic separation[1] of the serum proteins.

Histologic Procedures

All tissue samples collected for histologic examination with light microscopy were fixed in 10% formalin, trimmed, dehydrated, infiltrated, and embedded using conventional techniques. Three adjacent 6-micron sections were cut from each tissue block, 2 for tinctorial staining and 1 for immunofluorescent staining.

One section of each tissue was stained with hematoxylin and eosin (5) and examined to assess the histopathologic changes in tissues.

In an attempt to find a satisfactory tinctorial method for demonstration of Brucella in tissue sections, 3 staining procedures were compared in preliminary trials. Deparaffinized and hydrated guinea pig tissues containing Brucella, pure cultures of Brucella, purposely contaminated Brucella cultures, and blood samples containing Brucella were stained by the methods described by Stamp et al. (81), Christofferson and Ottosen (19), and Taylor (84). Ultimately, it appeared that the Taylor method, which is a modification of the Brown and

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[1]Spinco Model R Paper Electrophoresis System, Spinco Division, Beckman Instruments, Inc., Palo Alto, Calif.

Brenn Gram stain (6), was superior to the others. Therefore, one section from each tissue block was stained by that method and examined microscopically for the presence of Brucella-like forms.

In preliminary trials, various reagent preparation techniques for immunofluorescent staining were compared. Fluorescent antibody (FA) preparations made from both rabbit and canine anti-Br. canis and anti-Br. suis serums, as well as a commercial preparation,[1] were compared. Globulins were prepared by fractionation of serums with ammonium sulfate (18) and with ethanol (42) and labeled with fluorescein isothiocyanate (FITC) by adding the FITC to globulin and by a dialysis technique (20). All FA conjugates were dialyzed, absorbed with canine and murine liver powders (38), and passed through a Sephadex G-25[2] column to remove excess fluorescein and nonspecific material. Overall, FA preparations made from canine serums fractionated with ammonium sulfate and labeled by the dialysis procedure were found to be preferable. However, the staining of Br. canis was still considered unsatisfactory.

Numerous other preliminary trials were conducted to determine whether formalin-fixed, paraffin-embedded tissues were suitable for immunofluorescent examination and whether various

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[1]Bacto-FA Brucella abortus, Difco Laboratories, Detroit, Mich.

[2]Pharmacia Fine Chemicals Inc., Piscataway, N.J.

procedures would improve the quality and intensity of fluorescence of stained Brucella. In these preliminary trials it was determined that: (a) formalin fixation was not detrimental to FA staining of Brucella; (b) treatment with NH_4OH and Tween 80[1] enhanced fluorescent staining slightly; (c) 8 M urea would apparently dissociate antibody from antiserum-treated Brucella; (d) prestaining tissues with rhodamine improved the contrast between tissue cells and fluorescing material; (e) flazo orange counterstain (28) was not useful because FA-stained Br. canis still stained brightly with flazo orange, although FA-stained Br. suis did not; and (f) counterstaining of FA-stained Brucella with a rabbit anti-canine globulin FA, prepared similarly to other conjugates, enhanced the definition of stained organisms and improved the intensity of staining. The use of NH_4OH, Tween 80, urea and rhodamine was recommended by Prichard (74).

Briefly, the procedure used for immunofluorescent staining of deparaffinized, hydrated tissue sections was: 30 minutes in 1% Rhodamine B,[2] overnight in 8 M urea, 5 minutes in 1% NH_4OH, 5 minutes in 3% Tween 80, 1 hour at 37 C. in Br. canis or Br. suis FA, and 1 hour in canine globulin FA. Appropriate washings between steps were with distilled water or phosphate

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[1]Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2]National Aniline Division, Allied Chemical Corp., New York, N.Y.

buffered saline[1] (PBS). The stained sections were mounted with 90% glycerin in PBS and examined with a Leitz microscope equipped for fluorescence microscopy.

Tissue sections derived from Brucella-infected dogs were stained with the FA preparation specific for the infecting organism. Sections derived from control dogs were divided so that half were stained with Br. canis FA and half with Br. suis FA. The primary objective of the immunofluorescent staining was to determine the specific location of Brucella within tissues.

Cell cultures for immunofluorescent examination were fixed in formalin, passed through NH_4OH and Tween 80, stained with Br. canis FA or Br. suis FA, and counterstained with canine globulin FA as described.

Specimens of placental labyrinth (85) from dogs in the second canine experiment, medullary portions of lymph nodes from dogs in the third canine experiment, pure cultures of Br. canis, and guinea pig peritoneal macrophage cultures with and without Br. canis exposure were selected for electron microscopic examination. The tissue specimens were cut to 1 mm. size, fixed immediately in 2.5% glutaraldehyde (2 hours), washed with sodium cacodylate buffer at pH 7.4 (1 hour), post-fixed in 1% osmium tetroxide (1 hour), dehydrated through a

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[1]PBS = 1.177 Gm. Na_2HPO_4, 0.223 Gm. NaH_2PO_4, and 8.5 Gm. NaCl in 1000 ml. distilled water; pH = 7.5.

series of ethanols, and embedded in Epon (45). Thin sections were cut on an LKB Ultratome and stained with lead citrate and uranyl acetate (87). The macrophage cultures and Br. canis cultures were processed in a similar manner, except that fixed material was pelleted in 1% agar before embedding in Epon. The thin sections were examined in a Philips EM 200 electron microscope at 60 Kv.

RESULTS

Pathogenesis of *Brucella canis* and *Brucella suis* in Dogs

Clinical and postmortem findings

The most spectacular results of infection were observed in reproductive tracts of dogs infected with *Br. suis*. Reproductive disorders occurred in all sexually-mature bitches infected with *Br. suis*, but were not grossly detectable in those infected with *Br. canis* (Table 1).

The mammary glands of dog 322 (infected with *Br. suis*) began to enlarge at about 54 days after breeding. A serous secretion, which later became milky, could be expressed from the glands. Her abdomen was distended, although no pups were detected by abdominal palpation. At 57 days after breeding, she began building a nest. These signs were indicative of pseudocyesis (9). At 60 days she expelled copious amounts of mucopurulent, hemorrhagic exudate, but no placentas or pups, through the vagina. On postmortem examination 4 days later, the uterus still contained exudate, the uterine mucosa was markedly thickened, and corpora lutea were observed in the ovaries.

Signs of parturition were first observed in dog 325 at 60 days of gestation. Necropsy was performed at 62 days, after dystocia had been evident for 24 hours and manual extraction of a fetus had failed to relieve the dystocia. At

Table 1. The effect of *Brucella* infection on pregnancy in sexually mature dogs

Dog no.	Age (yr)	Post-breeding interval (days) At exposure	Post-breeding interval (days) At necropsy	Apparent outcome of pregnancy	Number of pups or fetuses Normal	Weak	Dead
Infected with *Br. canis*							
312	1	37	65[a]	Normal	10	0	0
328	3½	25	65[a]	Normal	4	0	0
376	3½	18	49	Normal	4	0	0
419	3½	10	49	Normal	6	0	0
421	1½	20	48	Nonpregnant	-	-	-
Infected with *Br. suis*							
322	4½	24	64	Pseudocyesis	-	-	-
325	1	38	62	Stillbirth-dystocia	0	0	6
377	2½	17	49	Probable abortion	2	1	1
378	1	21	50	Probable abortion	2	0	2
418	7	22	48	Abortion	0	1	2
Nonexposed controls							
302	4½	24[b]	70[a]	Normal	6	0	0
420	3	19[b]	48	Normal	8	0	0

[a]Necropsy performed on 312, 328, and 302 at 3 days postpartum.

[b]Days listed for 302 and 420 refer to their stage of gestation at the time principals were exposed.

postmortem examination the uterus contained blackened, partially decomposed placentas, pups, and exudate.

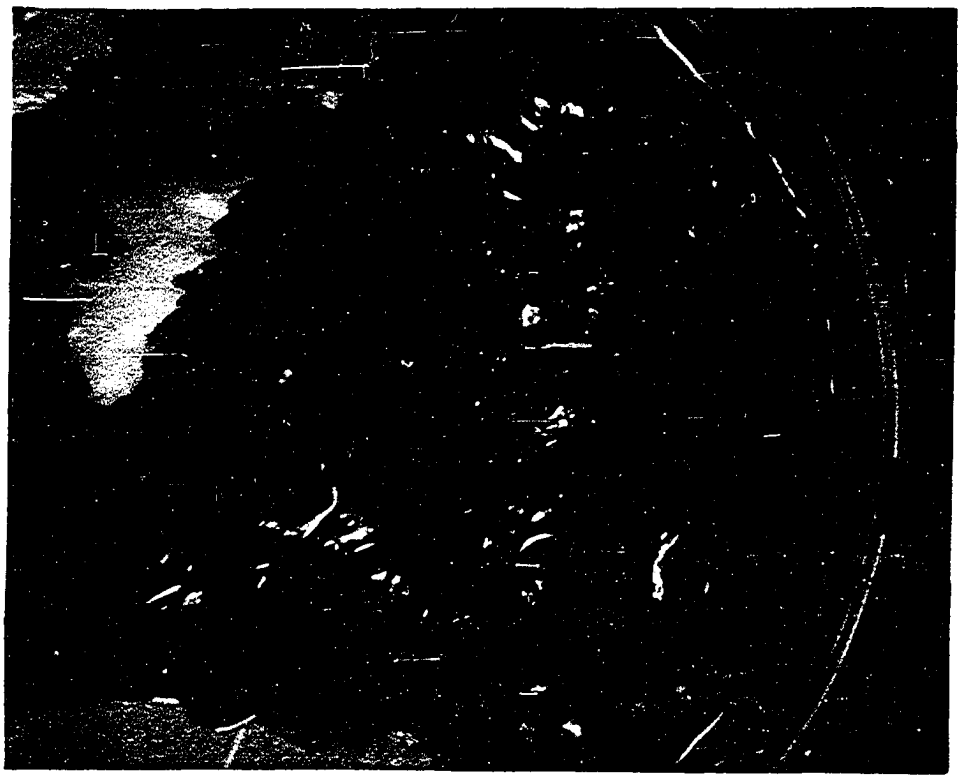
The severity and extent of reproductive involvement, due to Br. suis infection, in dogs 377, 378, and 418 were similar. Dogs 378 and 418 had a mucopurulent vaginal discharge on the day preceding necropsy. Dog 418 aborted one fetus before necropsy was begun. Upon postmortem examination the uteri of each of the above dogs contained both viable and nonviable fetuses and grossly pathologic placentas.

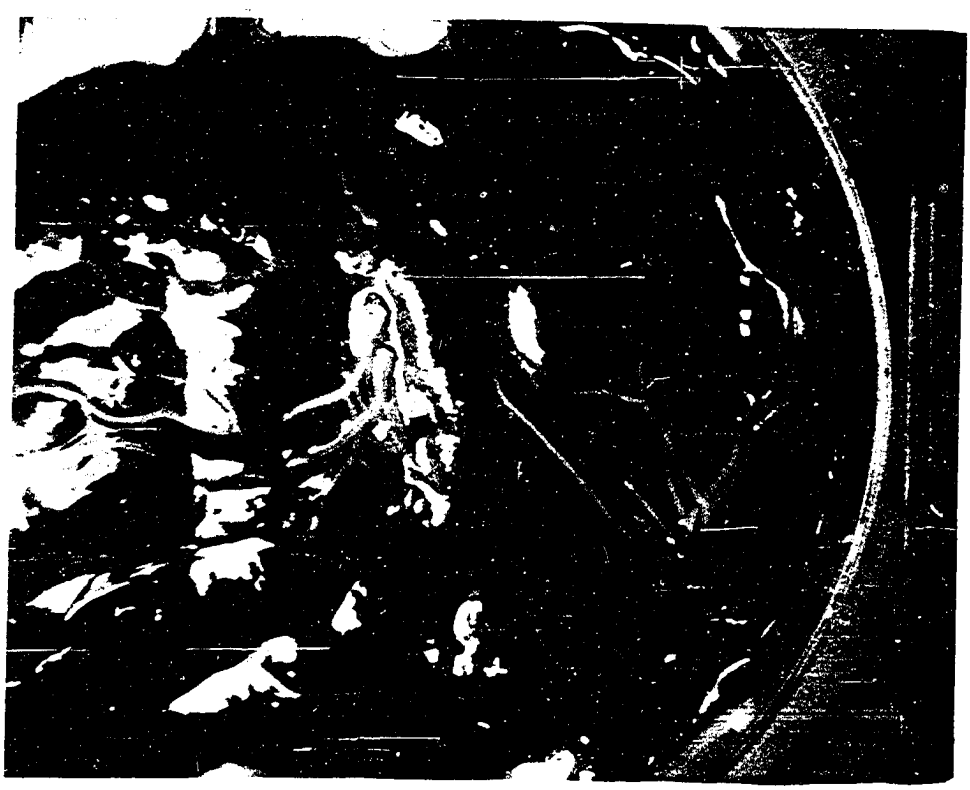
The placental changes consisted mainly of focal to diffuse yellowish discoloration of the placental labyrinth (Figures 1 and 2), separation of endometrium and placenta through the spongy layer with little traction, and cloudy, amber-colored allantoic and amniotic fluids. When the fetus was weak or nonviable, marked placental lesions were present. The placentas of 3 nonviable fetuses (1 from dog 378 and 2 from dog 418) had become detached from the endometrium before postmortem examination. Gross lesions were present in placentas of 2 viable fetuses--allantoic and amniotic fluid changes in one, and both fluid and focal placental labyrinth changes in the other.

Varying degrees of autolysis were present in all nonviable fetuses. These changes consisted of discoloration and peeling of skin, excess amber-colored subcutaneous, peritoneal, and thoracic fluid, and darkened, friable internal organs. The

Figure 1. Gross appearance of a normal placenta. Note the bright pink color. Control dog 420

Figure 2. Gross appearance of a degenerative placenta caused by Br. suis infection. Note the yellowish discoloration of the labyrinth and the brownish fluids. Dog 377





weights of weak and nonviable fetuses were always one third to two thirds that of their viable littermates.

There was nearly always a visible gradation of the severity of changes from one placenta and fetus to another. The alterations in one uterine horn were always more severe than in the other, and usually more severe at the distal end of a horn than near the body of the uterus.

Dog 480, a male, was the only dog that developed gross genital system lesions as a result of Br. canis infection (Table 2). At about 14 weeks postexposure (WPE) a unilateral epididymal enlargement was detected. At 17 WPE this dog developed scrotal dermatitis that persisted for 4 days. However, it was discovered that the floor of the dog's pen had been treated with a strong phenolic disinfectant[1] solution the day before the scrotal dermatitis was first observed. At necropsy dog 480 had enlargement and abscessation of the right epididymis, slight enlargement of the prostate gland, and the testes were small and firm.

Definite, palpable enlargement of subcutaneous lymph nodes occurred only in dogs 478 and 480 (Table 2). Enlargement of the nodes of these 2 dogs was detected by palpation only after 12 WPE. A few enlarged lymph nodes of other dogs were observed at postmortem (Table 2). In dogs infected with Br. canis the mandibular, suprapharyngeal, and superficial cervical nodes

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[1]Ves-phene, Vestal Laboratories, St. Louis, Mo.

Table 2. Gross pathologic changes observed at necropsy of dogs

Dog no.	DPE[a] at necropsy	Attributed to brucellosis: Reproductive tract lesions	Attributed to brucellosis: Enlarged lymph nodes (no.)	Attributed to brucellosis: Gm. lymph node/kg. body wt.[b]	Parasitisms: Adult forms	Parasitisms: Kidney lesions
Infected with Br. canis						
312	28	No	1	0.66	Ancylostoma	No
328	40	No	6	1.20	Ancylostoma	No
376	31	No	0	0.68	Dirofilaria Trichuris	No
419	39	No	0	1.23	Trichuris	No
421	28	No	4	1.34	Toxascaris	No
478[c]	140	No	All	2.26	Toxocara	Yes
480[c]	140	Yes	All	4.99	Toxocara	Yes
Infected with Br. suis						
322	40	Yes	3	1.21	None	No
325	24	Yes	0	N.D.[d]	None	No
377	32	Yes	2	1.14	None	Yes
378	29	Yes	1	1.13	Toxascaris	Yes
418	26	Yes	2	0.90	Trichuris	Yes
479[c]	49	No	0	1.40	Toxocara	Yes
481[c]	49	No	0	1.49	Toxocara Trichuris	Yes

[a]DPE = days postexposure; DPE of control dogs refers to DPE of principals.

[b]Total weight of lymph nodes collected divided by body weight.

[c]Dogs 477-481 were 4 months of age at exposure; 480 was male, others female.

[d]N.D. = not determined.

Table 2. (Continued)

Dog no.	DPE[a] at necropsy	Attributed to brucellosis: Reproductive tract lesions	Attributed to brucellosis: Enlarged lymph nodes (no.)	Attributed to brucellosis: Gm. lymph node/kg. body wt.[b]	Parasitisms: Adult forms	Parasitisms: Kidney lesions
Nonexposed controls						
302	46	No	0	0.79	Trichuris	No
420	29	No	0	0.99	Trichuris	Yes
477[c]	48	No	0	1.54	Toxocara	Yes

were most frequently affected, while in dogs infected with Br. suis, the internal iliac and axillary nodes were most often involved. Generalized lymphadenopathy was evident at necropsy only in the 2 dogs retained in the experiment for 20 weeks after exposure.

Clinical signs of disease were primarily limited to reproductive disorders. Anorexia was not observed. Pyrexia occurred only in dog 480. The fever first occurred at 67 days postexposure (DPE) and recurred periodically during the next 6 weeks. The fever episodes were mild, ranging from 39.9 to 40.3 C. and persisting for 1 to 5 days with interim 2- to 9-day periods when rectal temperatures were in the normal range (mean temperature during the first 50 DPE ±1 C.).

Parasitisms, diagnosed by periodic fecal examination during the experiments and by postmortem examination, were a common finding in the dogs (Table 2).

Nine of 12 dogs obtained from one supplier had multiple focal nodular lesions in their kidneys (Figure 3). Grossly, these lesions were yellow-white spots, about 1 mm. in diameter, located at or near the subcapsular surface of the kidney. Similar focal lesions were found on the surface of the liver of dog 479 and on the liver and lungs of dog 420.

Examinations for bacterial, mycotic, and parasitic etiology of the nodular lesions were conducted. Serums from the dogs did not contain agglutinins for Leptospira pomona, L. canicola, or L. icterohemorrhagiae. No bacterial or fungal

agents were detected by histologic examination of tissue sections stained with Taylor's modified Gram strain (84), Gridley's stain for fungi (5), and Periodic Acid-Schiff (McManus method) stain (5). Eventually, however, forms that were identified as nematode larvae were observed in a few lesions (Figure 4). The gross and microscopic appearance of the lesions was nearly identical to that described by Soulsby (78) as characteristic of Toxocara canis larval migration. Adult forms or ova of T. canis were often not detected in dogs in this study (Table 2), but an anthelmintic had been administered to all dogs before shipment to the laboratory. All lesions in kidneys, livers, and lungs of Brucella-infected and control dogs that were suggestive of parasitic etiology were attributed to Toxocara infection.

Additional gross lesions, not attributed to brucellosis or to parasitisms, were extensive adhesions of the peritoneum to abdominal organs in dog 377 and pneumoconiosis in dog 418.

Serologic findings

Serum antibody titers of dogs infected with Br. canis generally increased throughout the course of the experiment (Table 3). In dogs 478 and 480, killed at 20 WPE, serum antibody titers persisted at high levels throughout the 6- to 20-WPE period. Agglutinin titers from 800 to 12,800, ME titers from 400 to 1120, CF titers from 160 to 1280, and precipitins occurred during the 6- to 20-WPE period. There were no marked

Figure 3. Gross appearance of nodular lesions in the kidney. The capsule has been removed and the white spots in the surface of the renal cortex are visible. Control dog 477

Figure 4. Parasite larvae in cross-section within a lesion of the kidney shown in Figure 3. The larvae are approximately 20 microns in diameter. Periodic acid-Schiff stain, X400

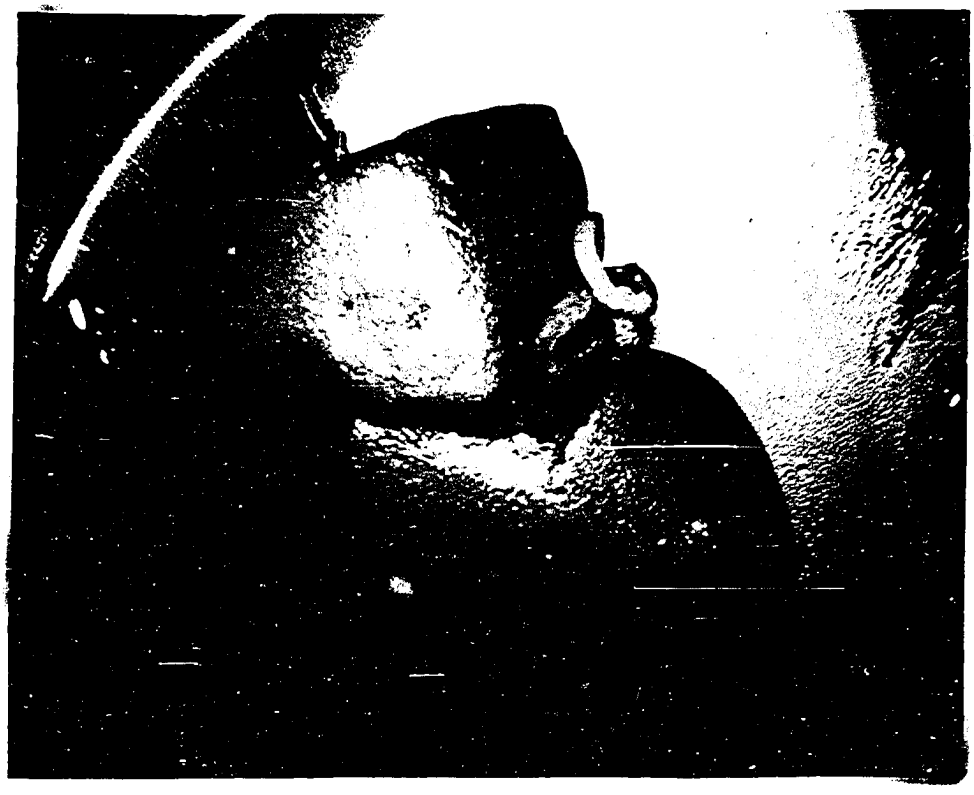


Table 3. Geometric mean antibody titers of serums from 7 dogs infected with Br. canis; determined with Br. canis antigens

Test	Days postexposure[a]									
	0	7	10	14	17	21	24	28	35	40-42
TA	-[b]	-	70(3)[c]	186(5)	263(5)	267(6)	296(7)	399(7)	436(4)	897(3)
ME	-	-	-	42(2)	70(3)	162(5)	148(7)	269(7)	382(4)	754(3)
CF	-	-	10(1)	18(4)	28(5)	32(7)	53(7)	100(7)	118(4)	269(3)
GDP	-	-	-	(3)	(4)	(5)	(6)	(7)	(4)	(3)

[a]Only 4 and 3 dogs are represented at 35 and 40 to 42 days, respectively.

[b]No antibodies detected in any serum.

[c]The mean includes only those serums in which antibodies were detected; the number in parentheses indicates the number of dogs in which serum antibodies were detected.

differences among the 4 tests in sensitivity for detection of antibodies. No Brucella antibodies were detected in serums of dogs before exposure, and nonexposed control dogs were uniformly test-negative throughout the experiments.

The ability of the GDP test with Br. ovis antigen to detect precipitins was equal to that of the Br. canis GDP test. No antibodies were detected in serums from Br. canis-infected dogs with the Br. abortus TA, ME, CF, and GDP tests.

Serums from pups born to dogs 312 and 328 were test-negative on the day of birth, but were usually test-positive on the succeeding 3 days. The ME titers of serums from pups that were 1 to 3 days old were always as high as TA titers of the same serums.

In contrast to the antibody production of Br. canis-infected dogs, serum antibodies in dogs infected with Br. suis usually appeared sooner after exposure, rapidly increased in concentration, and declined before 28 DPE (Table 4). The titers of all dogs, except 322 and 325, reached an apparent peak and receded before necropsy. Dog 325 (stillbirth and dystocia) was killed at 24 DPE. The TA, ME, and CF titers of dog 322 (pseudocyesis) had gradually increased to 2240, 1600, and 1280 by the time of necropsy (40 DPE). The TA, ME, and CF titers of dogs 479 and 481, which were killed at 49 DPE, were 140 to 200, 70, and 56 to 112 at 42 and 49 DPE. Precipitins were never detected in serums from dog 325. Also, precipitins were not detected in serums from dogs 479 and 481 at

Table 4. Geometric mean antibody titers of serums from 7 dogs infected with Br. suis; determined with Br. abortus antigens

Test	Days postexposure[a]									
	0	7	10	14	17	21	24	28	35	40-42
TA	-[b]	68(5)[c]	282(6)	486(7)	592(7)	564(7)	537(7)	449(6)	421(3)	421(3)
ME	-	-	84(2)	161(4)	130(5)	115(6)	168(6)	103(5)	105(3)	211(3)
CF	-	23(4)	67(5)	84(7)	90(7)	113(7)	122(7)	80(6)	90(3)	226(3)
GDP	-	(2)	(2)	(3)	(3)	(5)	(6)	(6)	(3)	(1)

[a]Only 6, 3, and 3 dogs are represented at 28, 35, and 40 to 42 days, respectively.

[b]No antibodies detected in any serum.

[c]The mean includes only those serums in which antibodies were detected; the number in parentheses indicates the number of dogs in which serum antibodies were detected.

6 and 7 WPE, although precipitins had been present in serums collected from 3 through 5 WPE.

Complement fixing antibodies were detected in 1 to 5 serums from each of 6 Br. suis-infected dogs with the Br. canis CF test, but the Br. abortus CF test titers were 4-fold to 32-fold higher on the same serums. Furthermore, serums with Br. abortus CF test titers of 160 to 320 were frequently test-negative with the Br. canis CF test. Only 1 dog (322) infected with Br. suis developed agglutinins that were detectable with Br. canis antigen. In this case the results of Br. abortus TA tests were always at least 16-fold higher than Br. canis TA tests. Serums from Br. suis-infected dogs were all test-negative with Br. canis ME and GDP tests.

Electrophoretic separation of serums collected from dogs at weekly intervals revealed few consistent alterations in serum protein composition as a result of Brucella infection. Alterations, manifested chiefly by increased concentration of gamma and beta globulins, did occur in serums from dogs 322, 478, and 480. The gamma + beta globulins in serums of dog 322 increased from 25 to 29 mg./ml. during 0 to 28 DPE to 38 mg., 45 mg., and 58 mg. at 35, 38, and 40 DPE, respectively. In dog 478 the globulin concentrations were 18 to 27 mg./ml. from 0 to 10 WPE, 30 to 38 mg./ml. from 11 to 16 WPE, and 22 to 25 mg./ml. from 17 to 20 WPE. The globulin concentrations in serums from dog 480 were 16 to 24 mg./ml. from 0 to 9 WPE and 29 to 38 mg./ml. during the last 11 weeks. The increases in

gamma and beta globulins were usually accompanied by slight increases in total serum protein and slight decreases in serum albumin. The mean gamma + beta concentrations of serums from infected dogs were less than that of control dogs at each week during the first 5 WPE.

Bacteriologic findings

Bacteremia was consistently present in all bred bitches after infection was established (Table 5). In dogs infected with Br. suis bacteremia occurred within one week after exposure. The numbers of circulating Br. suis rapidly increased, but soon receded back to low levels. Twenty of the 39 hemocultures that contained Br. suis required incubation and subculturing to detect the organisms. Conversely, bacteremia was seldom detected in Br. canis-infected dogs before 10 DPE, but the numbers of Br. canis per ml. of blood increased progressively throughout the course of the experiments. Only 4 of 38 hemocultures containing Br. canis required incubation and subculturing to detect the organisms.

There was a marked contrast in patterns of bacteremia between Br. suis and Br. canis infections in dogs exposed at 4 months of age (Table 6). Bacteremia was detected only once (at 2 WPE) in each dog infected with Br. suis. In the two Br. canis-infected dogs bacteremia was consistent, increased to enormous numbers of circulating organisms by 8 to 10 WPE, and persisted at extremely high levels until necropsy.

Table 5. Postexposure bacteremia in 5 bred bitches infected with Br. canis and in 5 bred bitches infected with Br. suis; mean colony forming units (CFU) per milliliter of blood[a]

	Days postexposure										
Infection	4	7	10	14	17	21	24	28	31	35	40
Br. canis	0	1(1)[b]	7(4)	36	43	48	57	110	161	93	1101
Br. suis	1(1)	5	39	8	17	10(4)	3	3	3	1	1

[a]A value of 1 CFU/ml. of blood was arbitrarily assigned when bacteremia could be detected only by incubation and subculturing of hemocultures.

[b]Numbers in parentheses indicate number of dogs with bacteremia when neither all nor none of the dogs in the group were bacteremic.

Table 6. Bacteremia in dogs exposed at 4 months of age to Br. suis (dogs 479 and 481) and Br. canis (dogs 478 and 480); data expressed in CFU/ml. of blood

Dog	Weeks postexposure									
	1	2	3	4	5	6	7	8	9	10
479	0	1	0	0	0	0	0			
481	0	4	0	0	0	0	0			
478	0	10	44	66	204	466	572	1440	988	3740
480	4	30	220	384	1520	2300	2640	5560	5000	10400
	11	12	13	14	15	16	17	18	19	20
478	1760	2940	1900	1400	1800	2560	2480	2360	2420	3220
480	2440	4140	1980	1380	2400	1280	1220	1640	1540	1500

Brucella suis or Br. canis was recovered intermittently from the vagina of most infected dogs from 17 DPE to necropsy. Brucella was isolated from the vagina of all infected sexually mature dogs on the day of abortion, parturition, and/or necropsy. Brucella suis was recovered from the nasopharynx of one dog (322) at 17 DPE.

Brucella was isolated from nearly all tissues that were examined from the sexually mature female dogs (Table 7). The numbers of organisms recovered from reproductive tracts of Br. suis-infected dogs were usually greater than were recovered from reproductive tracts of Br. canis-infected dogs.

Table 7. Results of bacteriologic examination of sexually mature female dogs

Tissue	*Br. canis* infection		*Br. suis* infection	
	Dogs positive/ dogs examined	Median[a] conc.	Dogs positive/ dogs examined	Median[a] conc.
Uterus	5/5	++++	5/5	+++++
Placentas	2/2	++++	4/4	+++++
Placental fluids	2/2	++	3/3	++++
Cervix	4/5	++++	5/5	+++++
Vagina	5/5	+++	5/5	++++
Ovaries	5/5	++	5/5	+++
Mammary glands	5/5	++	5/5	+++
Milk	2/2	++++	2/2	++++
Spleen	5/5	+++++	5/5	+++
Bone marrow	5/5	+++	5/5	+
Tonsils	5/5	++++	4/5	++
Liver	5/5	+++	5/5	++
Lungs	5/5	++	4/5	++
Kidneys	5/5	++	5/5	+
Adrenal glands	5/5	++	5/5	+++
Trachea	3/5	+	1/5	+
Bile	0/5	-	2/5	+
Urine	1/5	+	2/5	+
Brain	2/5	+	1/5	++
Cerebrospinal fluid	0/5	-	0/5	-

[a]Concentration of organisms determined by counting or estimating number of colonies/plate of medium: + = 1-30 colonies; ++ = 30-300 colonies; +++ = 300-3,000 colonies; ++++ = dense growth, but separate colonies; +++++ = confluent growth.

Conversely, other organs usually yielded greater numbers of Br. canis than Br. suis.

Brucella was recovered only from the lymph nodes and spleens of the two dogs (479 and 481) exposed to Br. suis at 4 months of age. In dog 478, a sexually immature female, Brucella canis was isolated from all those tissues listed in Table 7 except the uterus, cerebrospinal fluid, and bile. Brucella canis was recovered from the prostate gland, epididymides, and testes of dog 480, as well as from all other pertinent tissues listed in Table 7. The concentrations of organisms in the latter 2 dogs were particularly high (++++ to +++++) in the liver, spleen, and bone marrow of each.

All fetuses and pups from infected dams were infected (Table 8). Nearly all tissues examined from each Br. suis-infected fetus contained organisms, whereas the number of tissues containing organisms was variable in pups and fetuses infected with Br. canis. Only the liver and stomach contents of the stillborn pups from dog 325 (not included in Table 8) were examined. These tissues all contained Br. suis.

The lymph nodes of all infected dogs contained Brucella. The numbers of organisms recovered from nodes of Br. canis-infected dogs were, however, usually much greater than recovered from Br. suis-infected dogs (Table 9). The data obtained by examining separate lymph nodes of dog 480 did not indicate that particular nodes were infected while others were not (Table 10). However, dog 480 had experienced persistent

Table 8. Sites of recovery of *Brucella* organisms from pups and fetuses

Total no.	Subject	Organism	No. infected	No. from which organisms recovered from: Liver	Spleen	Lungs	Stomach contents	Heart blood	Kidneys	Thymus
14	pups	*Br. canis*	14	14	13	4	8	9	4	0
10	fetuses	*Br. canis*	10	6	5	8	5	3	4	3
11	fetuses	*Br. suis*	11	11	11	11	10	10	11	11

Table 9. Concentration of Brucella organisms in pooled lymph nodes of infected dogs; data expressed in the logarithm of colony forming units (CFU) per gram of tissue

Br. canis infection		Br. suis infection	
Dog	Log. CFU/gm.	Dog	Log. CFU/gm.
312	7.71	322	7.87
328	8.53	325	N.D.[a]
376	7.38	377	4.49
419	7.20	378	4.76
421	7.86	418	5.46
478	7.94	479	4.05
480	7.51	481	4.36
Mean	7.73	Mean	5.16

[a]All lymph nodes examined from dog 325 contained Br. suis, but CFU were not determined.

bacteremia for 19 weeks and had developed generalized lymph node enlargement before necropsy. Bacteriologic examination of lymph nodes removed surgically from dogs exposed at 4 months of age provided the same general relationships as nodes removed at necropsy (Table 11).

Generally, Brucella was the only bacteria recovered. Streptococcus uberis was isolated from the kidneys, urine, and right epididymis of dog 480 and the kidneys, urine, and tonsils of dog 478. Much greater numbers of streptococci than Br. canis were recovered from the right kidney of dog 480.

Table 10. Distribution and concentration of Br. canis among lymph nodes of dog 480

Lymph node	Log. CFU/gm.	Lymph node	Log. CFU/gm.
Suprapharyngeal	7.42	Bronchial	7.66
Parotid	6.97	Hepatic	7.57
Mandibular	7.53	Mesenteric	5.92
Superficial cervical	7.29	Internal iliac	7.50
Sternal	8.48	Superficial inguinal	7.48
Axillary	7.04	Popliteal	7.38

Table 11. Concentration (log. CFU/gm.) of Brucella in lymph nodes surgically removed from dogs exposed at 4 months of age

Week post-exposure	Lymph node	Br. canis 478	Br. canis 480	Br. suis 479[a]	Br. suis 481[a]
4	L. sup. cervical	6.95	7.11	4.53	4.83
8	R. sup. cervical	7.66	7.98	N.D.[b]	N.D.
12	L. popliteal	7.98	8.39	N.D.	N.D.

[a]Killed at 7 weeks postexposure.

[b]N.D. = not determined.

No Brucella was recovered from the nonexposed control dogs during the course of the experiments or at necropsy.

Histologic findings

Histopathologic alterations in infected dogs were observed more often in reproductive and lymphatic systems than in other tissues (Table 12). Microscopic lesions were observed frequently in individual lymph nodes, spleens, and tonsils from nonexposed control dogs, but changes attributed to infection were of greater extent and severity than observed in controls; consequently, the distinguishing features were mostly quantitative, rather than qualitative. The extent and intensity of immunofluorescence in sections stained with FA were rated from + to ++++, but there was some nonspecific fluorescence in all tissues, and the specificity of staining was often questionable when only minimal fluorescence (+) was observed.

Reproductive system Placentas from dogs infected with Br. suis contained marked histopathologic changes. The most consistent changes were observed in the deep glandular layer and in the spongy layer and base of the placental labyrinth. However, the lamellae of the placental labyrinth, supraglandular layer, and myometrium of some placentas were also severely involved.

Lesions in the myometrium appeared to be an extension from inflammatory changes in the deep glandular layer. The myometrial changes consisted of edema and interstitial

Table 12. Sites of histopathologic changes, attributable to Brucella infection, in dogs

Dog no.	Exposure (days post-breeding)	Necropsy (days post-exposure	Tissues affected					
			Genital tract	Mammary glands	Lymph nodes	Spleen	Liver	Kidneys
Infected with Br. canis								
312	37	28	-	-	-	-	-	-
328	25	40	-	-	+	-	-	-
376	18	31	-	-	-	-	-	-
419	10	39	+	+	+	-	+	-
421	20	28	-	NA[a]	+	-	-	-
478	NA	140	-	NA	++[b]	+	+	-
480	NA	140	++	NA	++	+	+	+
Infected with Br. suis								
322	24	40	++	+	+	-	+	-
325	38	24	++	+	-	-	-	-
377	17	32	++	-	-	-	+	-
378	21	29	++	-	+	+	+	-
418	22	26	++	+	+	+	+	-
479	NA	49	-	NA	-	-	-	-
481	NA	49	-	NA	-	-	-	-

[a]NA = not applicable because of nonpregnancy or sexual immaturity.

[b]++ = changes severe or extensive.

infiltration with lymphoid cells, neutrophils, and macrophages (Figure 5).

The reaction in the deep glandular layer was often suppurative. There were numerous interstitial neutrophils and the lumen of the glands were often filled with suppurative exudate. Coagulative necrosis and erosion of glandular epithelium were often observed. In the most severely affected placentas, there were foci where glands were obliterated and macrophages, lymphocytes, plasma cells, and neutrophils filled the area (Figure 6). Immunofluorescence, when observed, in the deep glandular layer was minimal (+) to moderate (++) and was usually located within the glands.

The supraglandular connective tissue in a few placentas was infiltrated with lymphoid cells and neutrophils. The infiltrations were diffuse with focal accumulations (Figure 7).

The glands of the spongy layer consistently had areas of focal to diffuse coagulative necrosis (Figure 8). Epithelial cells that had been sloughed were often observed in the lumen. The most severely involved areas were at the junction of the spongy layer and the base of the placental labyrinth. Large accumulations of neutrophils were often observed in this area and necrosis of the maternal glandular tissue and chorionic villi was prominent. Extracellular colonies of bacteria and intracellular bacteria, within the chorionic epithelium, were observed in this area (Figures 9 and 10). Immunofluorescent staining and Gram stains indicated that the majority of the

Figure 5. Inflammation in the myometrium and deep glandular layer. There is cellular infiltration in the interstitial tissue and exudate in the lumen of the glands. Dog 378, Br. suis infection. Hematoxylin and eosin stain, X100

Figure 6. The myometrium and deep glandular layer where glands have been obliterated (upper left) and replaced by inflammatory cells. Dog 418, Br. suis infection. Hematoxylin and eosin stain, X100

Figure 7. Inflammatory cell infiltration into the supraglandular connective tissue. There is also a suppurative focus (center) of the supraglandular layer, and inflammation extends into the spongy layer of the placenta (upper). Dog 418, Br. suis infection. Hematoxylin and eosin stain, X100

Figure 8. Coagulative necrosis of glands in the spongy layer (left). The normal glandular tissue (right) is attached to the supraglandular layer. Dog 418, Br. suis infection. Hematoxylin and eosin stain, X200

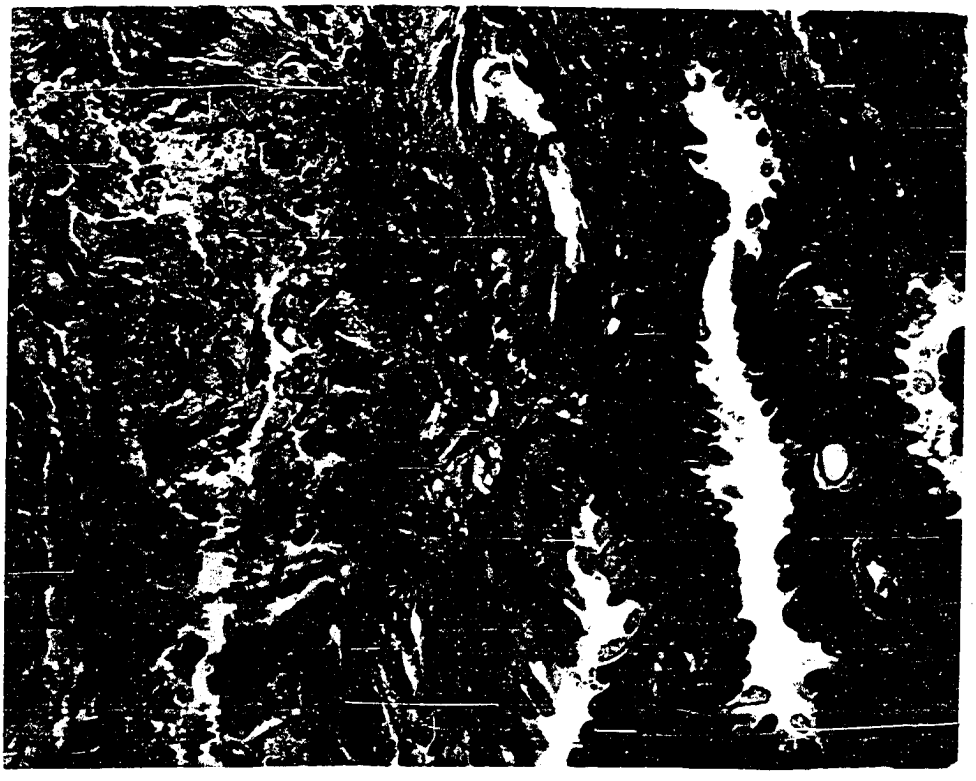
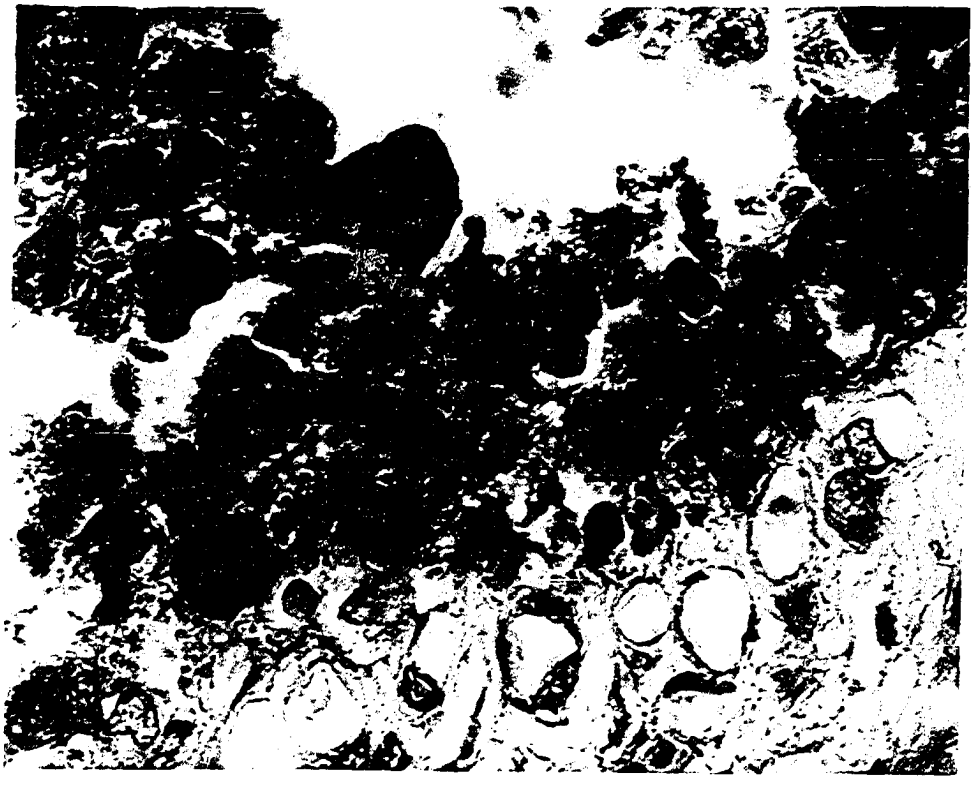
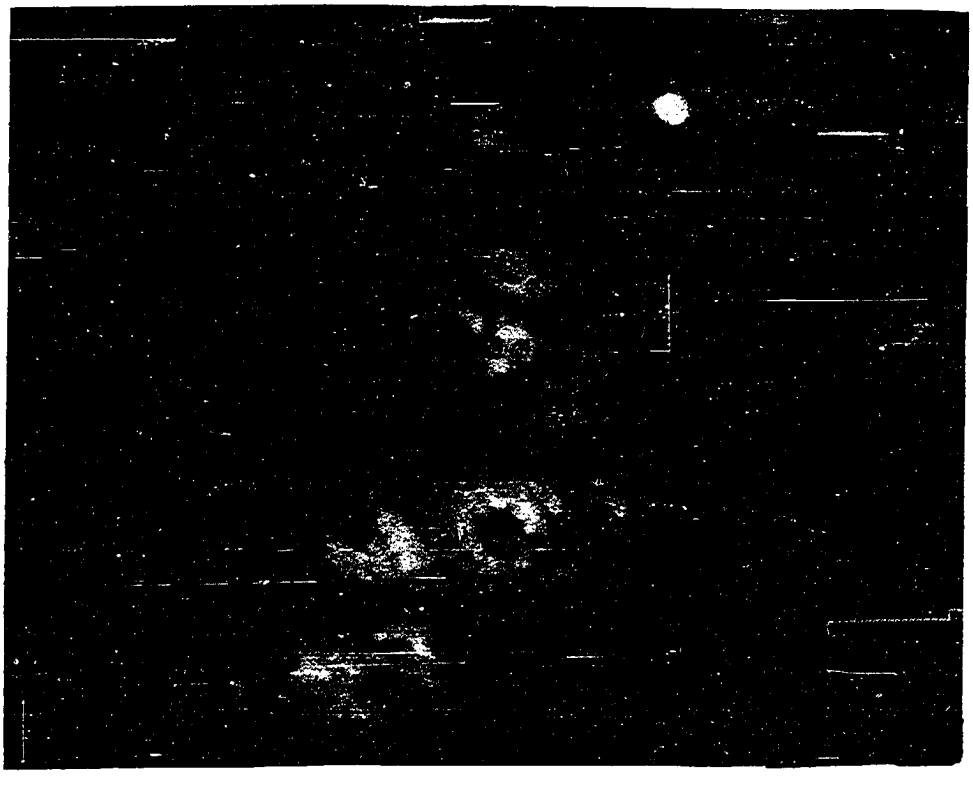


Figure 9. Intracellular and extracellular bacteria in a chorionic villus. Many of the trophoblastic epithelial cells have ruptured or degenerated. Dog 418, Br. suis infection. Taylor's modified Gram stain, X700

Figure 10. Immunofluorescence of Br. suis in chorionic epithelium of a placenta from dog 377. Brucella suis FA and canine globulin FA, X560





organisms in placentas of Br. suis-infected dogs were concentrated in this area.

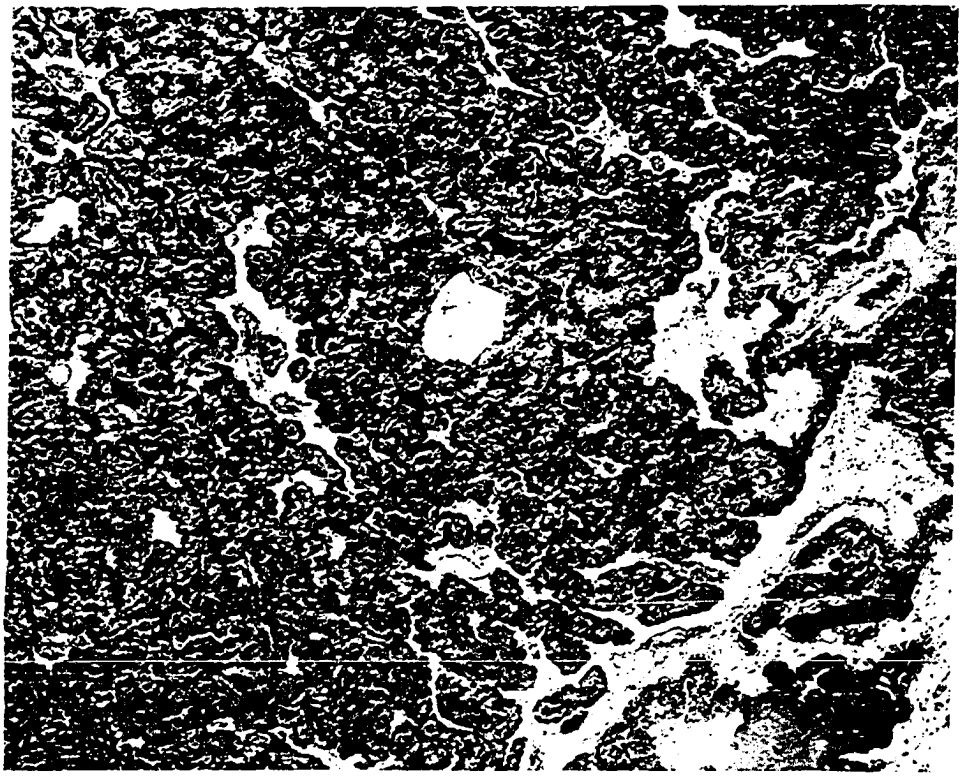
The lamellae of the placental labyrinth in some sections had large focal degenerative areas (Figures 11 and 12). These changes were characterized as a fibrinoid degeneration of the maternal capillary endothelium and syntrophoblast. The changes in the lamellae were not accompanied by the infiltration of inflammatory cells. Immunofluorescence in the lamellae of the placental labyrinth was never rated greater than ++. The ultrastructure of cells examined from this area was either normal or in an advanced state of necrosis (Figures 13 and 14).

No histopathologic changes were detected in the allantochorion or in amniotic membranes.

Microscopic lesions in placentas from the pregnant Br. canis-infected dogs were mild and inconsistent. Two placentas (of 6) from dog 419 contained a few areas of diffuse lymphocytic and neutrophilic infiltration in interstitial tissue of the deep glandular layer. There were also small suppurative foci in the supraglandular connective tissue of one of the placentas. Immunofluorescence (+ to ++) was observed in 2 placentas each from dogs 376 and 419. The fluorescence was distributed more diffusely throughout these placentas than in Br. suis-infected placentas. All thin sections of Br. canis-infected placental labyrinths observed with the electron microscope were normal.

Figure 11. Normal placental labyrinth with normal lamellae. At the lower right are the terminal ends of chorionic villi. Control dog 420. Hematoxylin and eosin stain, X40

Figure 12. Degenerative changes in the lamellae of the placental labyrinth. There are bacterial colonies and large masses of neutrophils at the lower right. Compare with Figure 11. Dog 377, Br. suis infection. Hematoxylin and eosin stain, X40



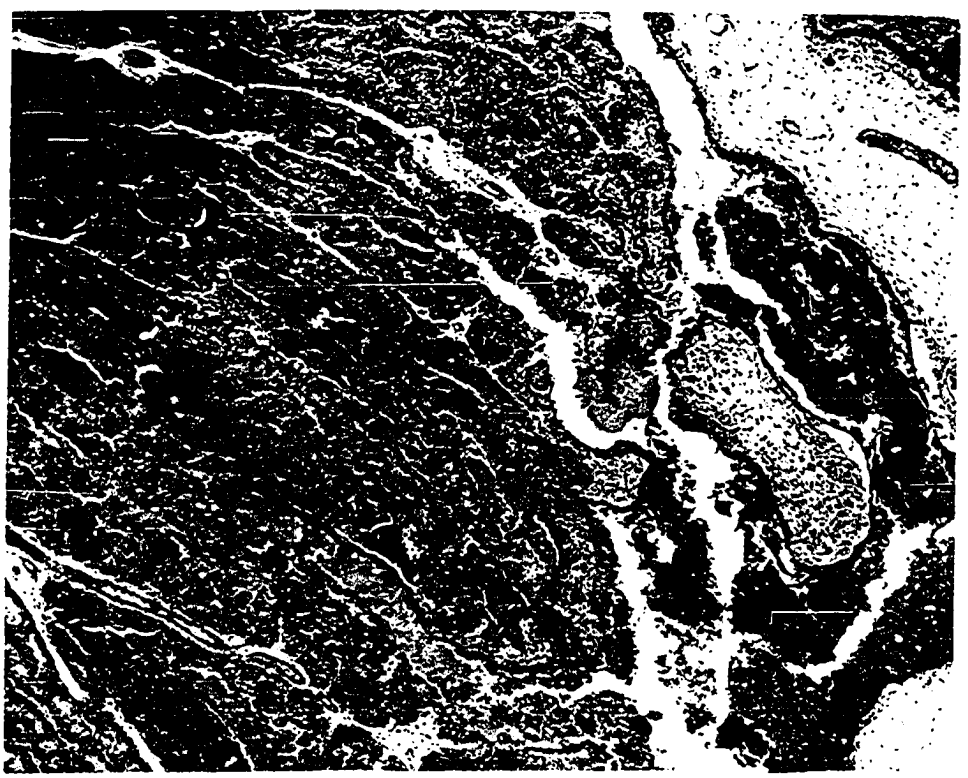
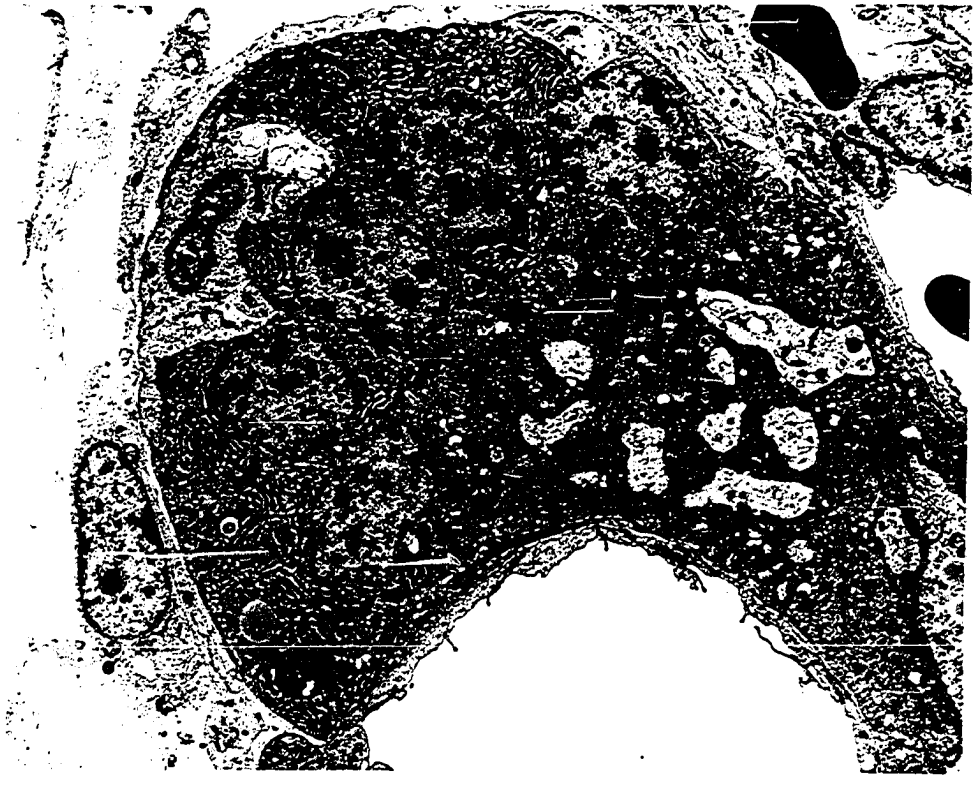


Figure 13. A normal trophoblastic syncytial cell from lamellae of placental labyrinth, bordered by capillary endothelium. Control dog 420. Lead citrate and uranyl acetate stain, X3,900

Figure 14. Degenerative trophoblastic epithelium from lamellae of placental labyrinth. There is disruption of the cytoplasmic architecture and margination of chromatin in the nuclei, but cell membranes are still discernible. Dog 418, Br. suis infection. Lead citrate and uranyl acetate stain, X9,000



Fetuses and pups Postmortem autolysis was present in the internal organs of all nonviable fetuses from Br. suis-infected dams. There were no microscopic lesions in livers, lungs, spleens, kidneys or thymuses of viable fetuses and pups. Fluorescence was observed in some sections of fetal organs stained with FA. Moderate fluorescence, both intracellular and extracellular, was seen in all organs examined from 2 fetuses of dog 418 and in the lung of one fetus from dog 377. Of the other 8 fetuses from Br. suis-infected dogs, minimal FA staining was present in 2 lungs, 1 thymus, 1 liver, and 2 spleens. Of the 24 Br. canis-infected fetuses and pups, minimal FA staining was seen in 8 livers, 4 lungs, 4 spleens, and 1 thymus.

Female genital tracts Diffuse cystic endometritis was observed in the uterus of dog 322 (pseudocyesis) and there was minimal intraepithelial cell fluorescence in FA stained sections. The endometrium in dog 325 (dystocia) was also hyperplastic and +++ fluorescence was observed. There were dense, diffuse infiltration of lymphocytes, macrophages and neutrophils in the lamina propria of uteri from these 2 dogs.

Sections from nonpregnant uteri and from placental attachment areas in uteri of postpartum Br. canis-infected bitches were all indistinguishable from analogous sections of control dogs. No microscopic lesions were observed in ovaries. Other parts of the genital tracts were not examined.

Mammary glands Multiple focal acute mastitis was found in 3 dogs (322, 325, and 418) infected with Br. suis and one dog (419) infected with Br. canis. The lesions were characterized as interstitial infiltrations of macrophages, neutrophils, lymphocytes, and plasma cells; oftentimes an entire lobule was diffusely involved, while adjacent lobules were unaffected (Figure 15). Minimal FA staining, usually in interstitial areas, was observed in some sections from each of 4 Br. suis-infected and 4 Br. canis-infected dogs.

Male genital organs The genital organs of dog 480, the only male used, had marked microscopic lesions. Complete aspermia was the outstanding testicular abnormality (Figure 16) in this dog which was 9 months of age at necropsy. Spermatagonia were present in all seminiferous tubules, but there were no other indications of spermatogenesis. There was no inflammatory response in the testes. There was a lymphocytic interstitial epididymitis in the left epididymis and extending into interstitial tissue surrounding the vas deferens. The cellular infiltration was diffuse with focal accumulations (Figure 17). In the right epididymis there was an extensive suppurative reaction (Figure 18), but there were also lymphocytic foci bordering the areas of liquefactive necrosis. Streptococcus uberis, as well as Br. canis, had been isolated from this epididymis. Multiple focal interstitial lymphocytic prostatitis was also observed. In some areas the interstitial accumulations had obliterated prostatic ducts.

Figure 15. Acute focal mastitis. The affected lobule (upper left) is adjacent to normal lobules (lower right). Dog 418, Br. suis infection. Hematoxylin and eosin stain, X100

Figure 16. Aspermia in a testis. The cells lining the tubules are mainly spermatogonia. There is no discernible inflammation. Dog 480, Br. canis infection. Hematoxylin and eosin stain, X100

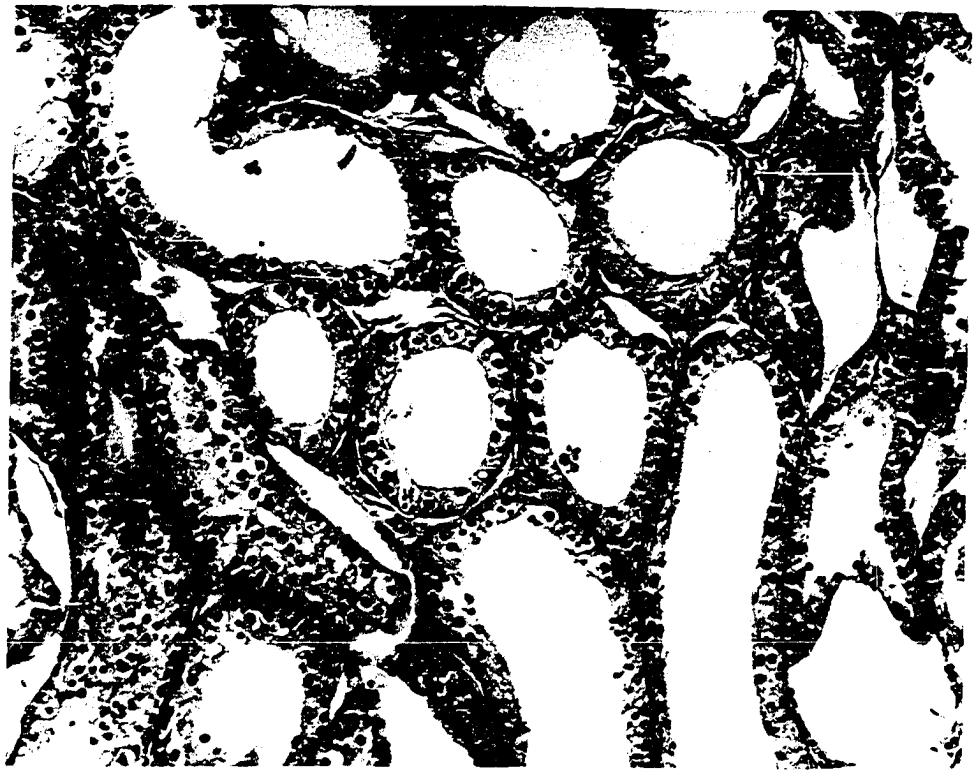
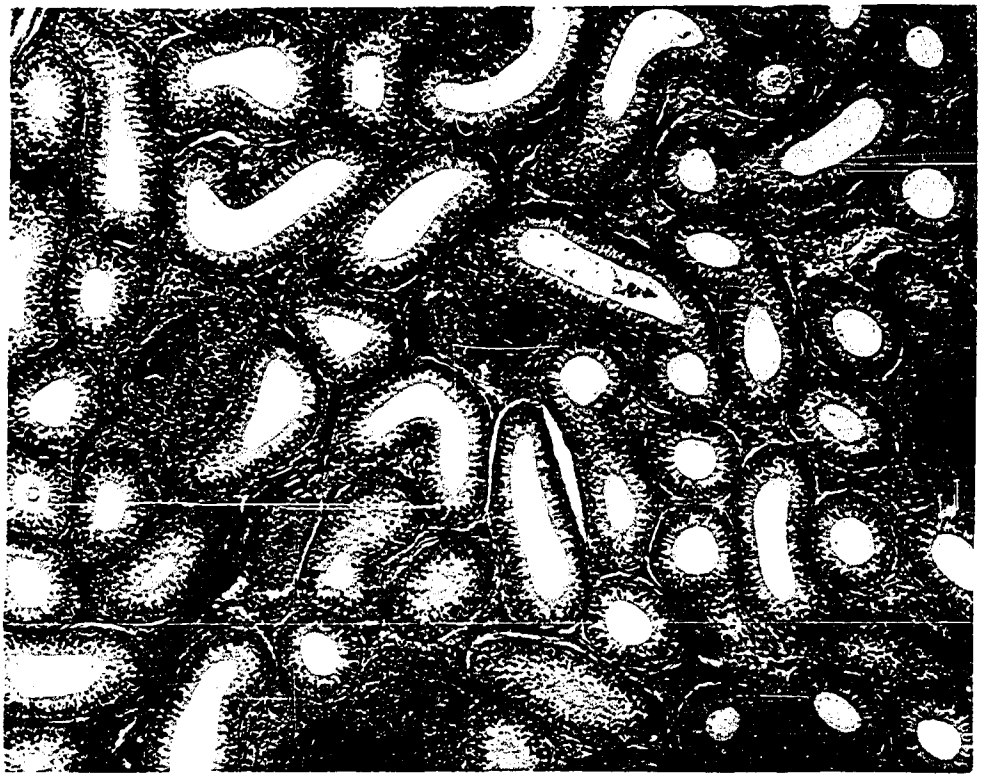


Figure 17. Multiple focal interstitial lymphocytic accumulations caused by Br. canis infection in the left epididymis of dog 480. Hematoxylin and eosin stain, X40

Figure 18. Diffuse suppurative epididymitis in the right epididymis of dog 480. There is a large area of liquefactive necrosis (right and lower). Brucella canis and Strep. uberis were recovered from this epididymis. Hematoxylin and eosin stain, X40



Lymphatic system Histopathologic changes in lymph nodes from dogs infected with Br. suis were not distinguishable from those of dogs infected with Br. canis, when the dogs were necropsied at similar postexposure periods.

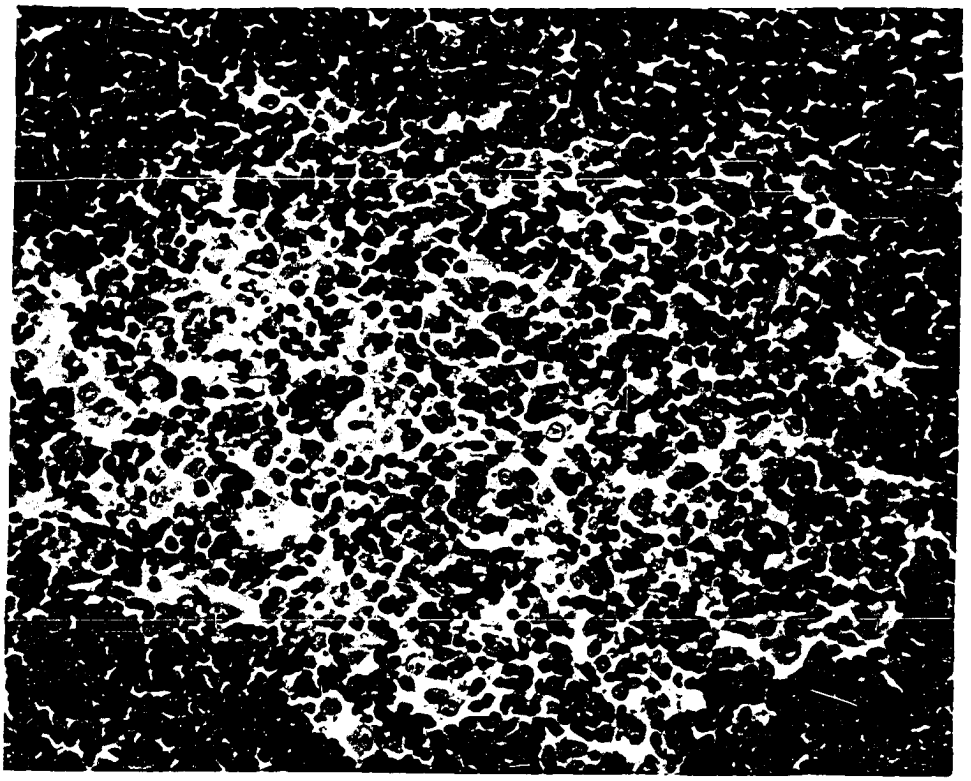
Proliferative changes, particularly lymphoid hyperplasia, were the characteristic feature of lymph node alterations. The centers of lymphoid nodules were often devoid of mature lymphocytes and mitoses were prominent (Figure 19). Occasionally, in nodes from dogs 478 and 480 (killed at 20 WPE), entire nodules were devoid of lymphocytes, contained foci of coagulative necrosis, and were infiltrated with eosinophils and neutrophils (Figure 20). It appeared that the lymphocytogenic function of these nodules was exhausted.

A prominent feature of some lymph nodes was a marked plasmacytosis. In these nodes the predominant cells within the sinuses were plasma cells (Figure 21). In fact, the cellular content of many sinuses consisted of 80 to 90% plasma cells. Neutrophils, diffuse with focal accumulations, were observed in many nodes. Marked edema was seen in some nodes. Reticular hyperplasia was evident in a few nodes.

Bacteria were not observed in lymph node sections stained with the Gram stain. Fluorescent areas were seldom seen in FA stained sections. Nonspecific staining occurred in all sections from both infected and control dogs. However, minimal fluorescence (+) was recorded for 7 of 36 sections from Br. suis-infected dogs and for 7 of 41 sections from Br. canis-infected

Figure 19. Hyperplastic germinal center in a lymph node. The center of the nodule is nearly devoid of lymphocytes and there are numerous mitotic figures. Dog 480, Br. canis infection. Hematoxylin and eosin stain, X200

Figure 20. Exhausted lymphoid nodule (right center) from lymph node of Br. canis-infected dog 478. There is coagulative necrosis in the center and infiltration of neutrophils and eosinophils into the nodule. The surrounding germinal centers are hyperplastic. Hematoxylin and eosin stain, X40



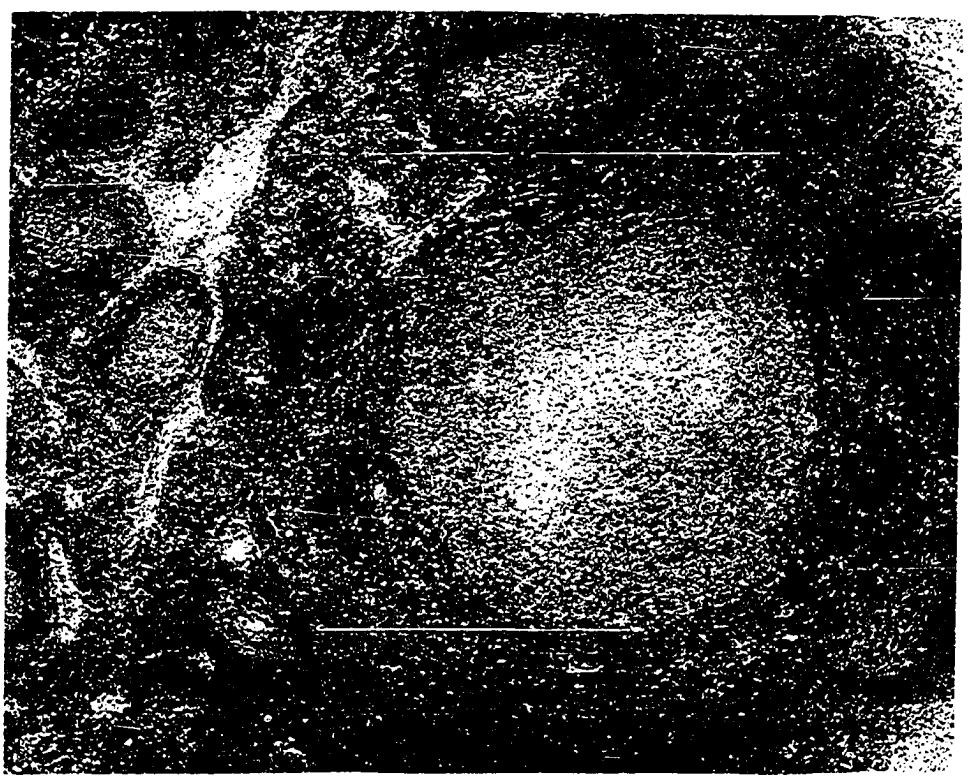
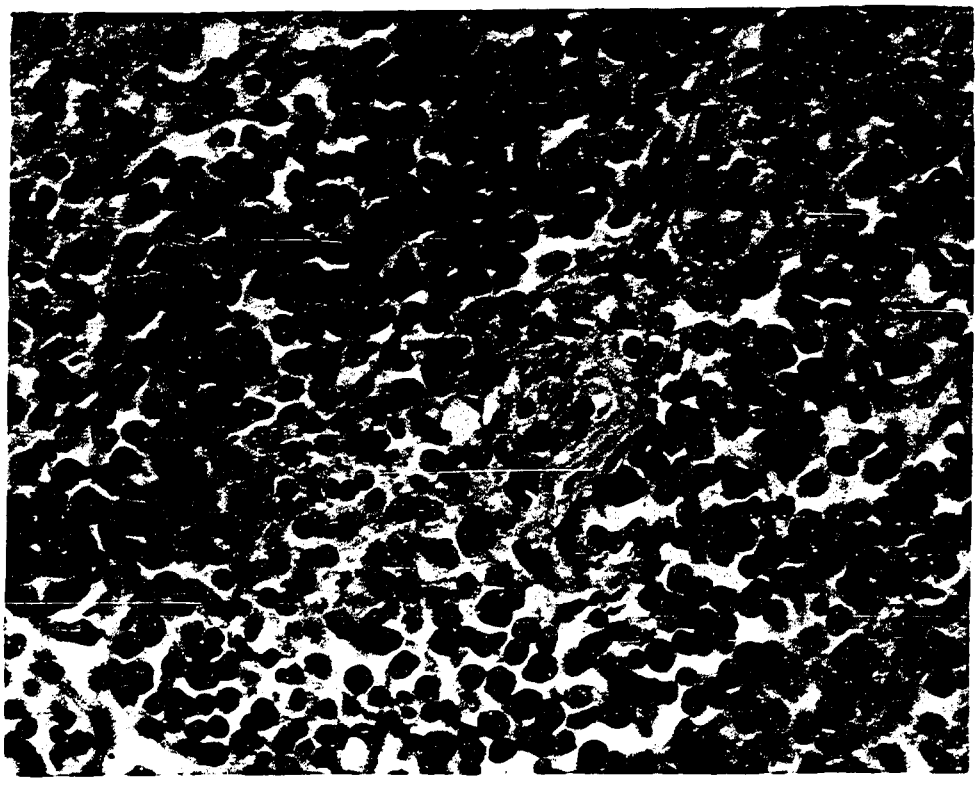
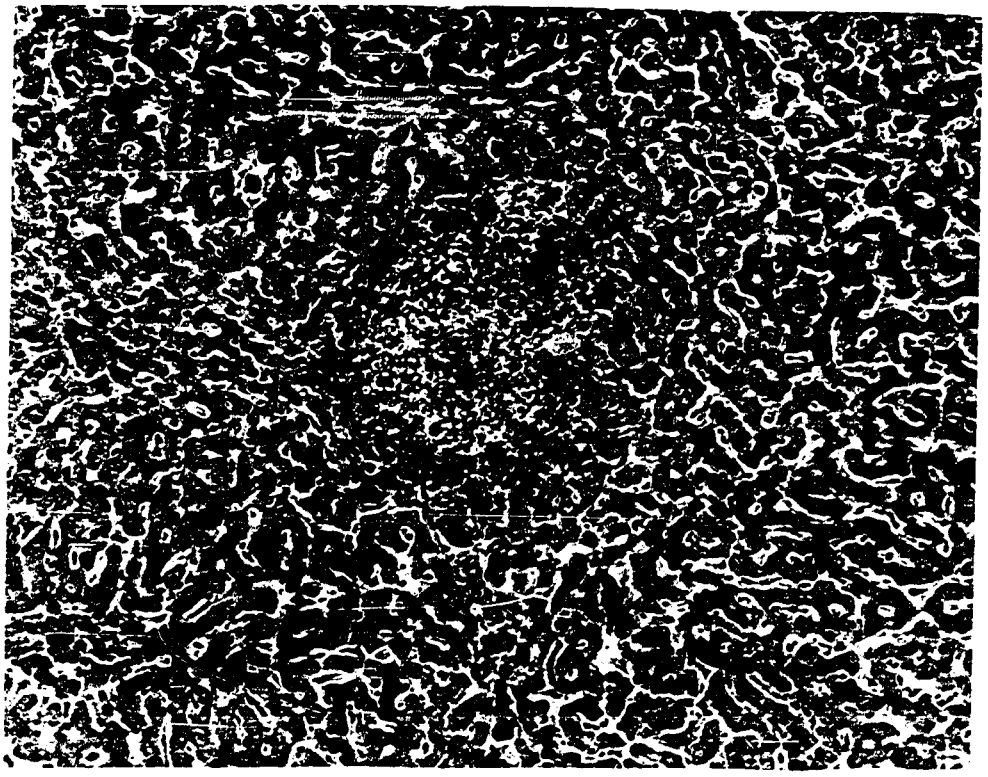


Figure 21. Plasmacytosis in a lymph node. The majority of cells in the sinuses are plasma cells. Dog 418, Br. suis infection. Hematoxylin and eosin stain, X400

Figure 22. Necrotic focus in a liver. The surrounding and infiltrating cells are erythrocytes, macrophages, lymphocytes, and neutrophils. Dog 378, Br. suis infection. Hematoxylin and eosin stain, X100





dogs. The fluorescence was located intracellularly and extracellularly in reticular tissue.

Sections of lymph nodes removed surgically at 4 and 8 WPE were indistinguishable from each other and did not reveal definite sequential changes. However, nodes removed at 12 WPE from dogs 478 and 480 contained marked changes, similar to those already described. Electron microscopy of medullary areas of the latter nodes revealed large proportions of plasma cells.

Spleens from infected dogs were usually not clearly distinguishable from those of control dogs. All spleens had evidence of lymphoid depletion. In a few infected dogs, slight hyperplasia of white pulp was evident. Megakaryocytosis was prominent in all spleens, but was often more prevalent in those from infected dogs.

Other tissues Multiple focal hepatitis was observed in 4 dogs infected with Br. suis. The changes consisted of focal accumulations of macrophages and lymphocytes or foci of coagulative necrosis surrounded by lymphoid cells, macrophages, neutrophils, and erythrocytes (Figure 22). Lymphocytic infiltration of hepatic triads was also a common finding. Histopathologic changes were also observed in livers of 3 dogs (419, 478, and 480) infected with Br. canis. The changes consisted of small focal accumulations of neutrophils, lymphocytes, macrophages, and plasma cells. The accumulations occurred throughout the sinusoids, but tended to be concentrated

near central veins and hepatic triads. The reaction was somewhat more diffuse and less marked than in Br. suis-infected livers and there was no necrosis observed.

The kidneys of dog 480, but not other dogs, had lesions that were not attributable to parasitic migration. In both kidneys there were small clusters of plasma cells around the tubules. In the right kidney there was a diffuse suppurative reaction in the medulla. There were also large collections of macrophages, neutrophils, lymphocytes, eosinophils, and plasma cells at the corticomedullary junction. Large numbers of Strep. uberis and smaller numbers of Br. canis had been isolated from the right kidney.

There were no microscopic lesions, clearly attributable to brucellosis, observed in lungs, tonsils, or adrenal glands.

Pathogenecity of Brucella canis for Large Animals

Cattle

None of the cattle exposed to Br. canis developed clinical signs of disease. The heifer which was killed at 28 DPE was pregnant with twin fetuses, both of which were normal. The 5 that were kept until after parturition gave birth to live, healthy calves.

A mild serologic response occurred after exposure (Table 13). Peak titers with TA, ME, and CF tests were 140, 70, and 28, respectively. No precipitins were detected. Whey samples from all heifers had titers of 25 to 70 with the

Table 13. Geometric mean Brucella canis antibody titers in serums of 6 cattle

Test	Weeks postexposure[a]										
	0	1	2	3	4	6	8	10	12	16	20
TA	50(3)[b]	70	84	89	94	81	84	84(4)	66	60	59
ME	--[c]	--	25(1)	61(5)	40(5)	50(4)	25(1)	--	--	--	--
CF	--	--	--	15	17(2)	--	--	--	--	--	--
GDP	--	--	--	--	--	--	--	--	--	--	--

[a]Only 5 cattle remained in the experiment after 4 weeks postexposure.

[b]Only titers of serums in which antibodies were detected used to calculate means; numbers in parentheses indicate number of animals in which serum antibodies were detected.

[c]No antibodies detected in any serum.

Br. canis TA test, but all were test-negative with the Br. canis ME test. Serum from one calf, 24 hours old at the time of blood collection, had a TA titer of 70 that was not mercaptoethanol-resistant. Serums of 3 heifers had TA test titers of 25 to 70 before exposure. Brucella abortus serum agglutinin titers (not mercaptoethanol-resistant) of 25 to 50, that were not considered to be associated with exposure to Brucella, occurred in 3 cattle at various times during the experiment.

Brucella canis was not isolated from hemocultures or tissues of any cattle. At necropsy, there were no gross lesions indicative of Brucella infection; consequently, none of the tissues were examined for histopathologic changes.

Swine

No clinical signs of disease were observed in swine exposed to Br. canis. All the gilts farrowed normally, giving birth to live healthy pigs. Furthermore, no gross lesions attributable to Brucella infection were observed in swine at necropsy.

There was little or no serologic response as a result of exposure to Br. canis (Table 14). Antibodies were detected only with the TA test, and the highest titer was 200 (in only 2 serums). Eleven of the 12 swine had agglutinin titers, from 25 to 100, on the day of exposure. No Br. canis agglutinins were detected in serums from these swine at 4 months pre-exposure. Serums of newborn pigs did not contain Br. canis

Table 14. Geometric mean Brucella canis antibody titers in serums of 12 swine

Test	Weeks postexposure[a]										
	0	1	2	3	4	5	6	7	8	10	12
TA	50(11)[b]	52(11)	69	100	79	73	86(9)	80	52(7)	77(4)	37(3)
ME	--[c]	--	--	--	--	--	--	--	--	--	--
CF	--	--	--	--	--	--	--	--	--	--	--
GDP	--	--	--	--	--	--	--	--	--	--	--

[a]There were only 10 swine remaining in the experiment at 5 and 6 weeks, 8 swine at 7 and 8 weeks, 6 swine at 10 weeks, and 4 swine at 12 weeks.

[b]Only titers of serums in which antibodies were detected used to calculate means; numbers in parentheses indicate number of animals in which serum antibodies were detected.

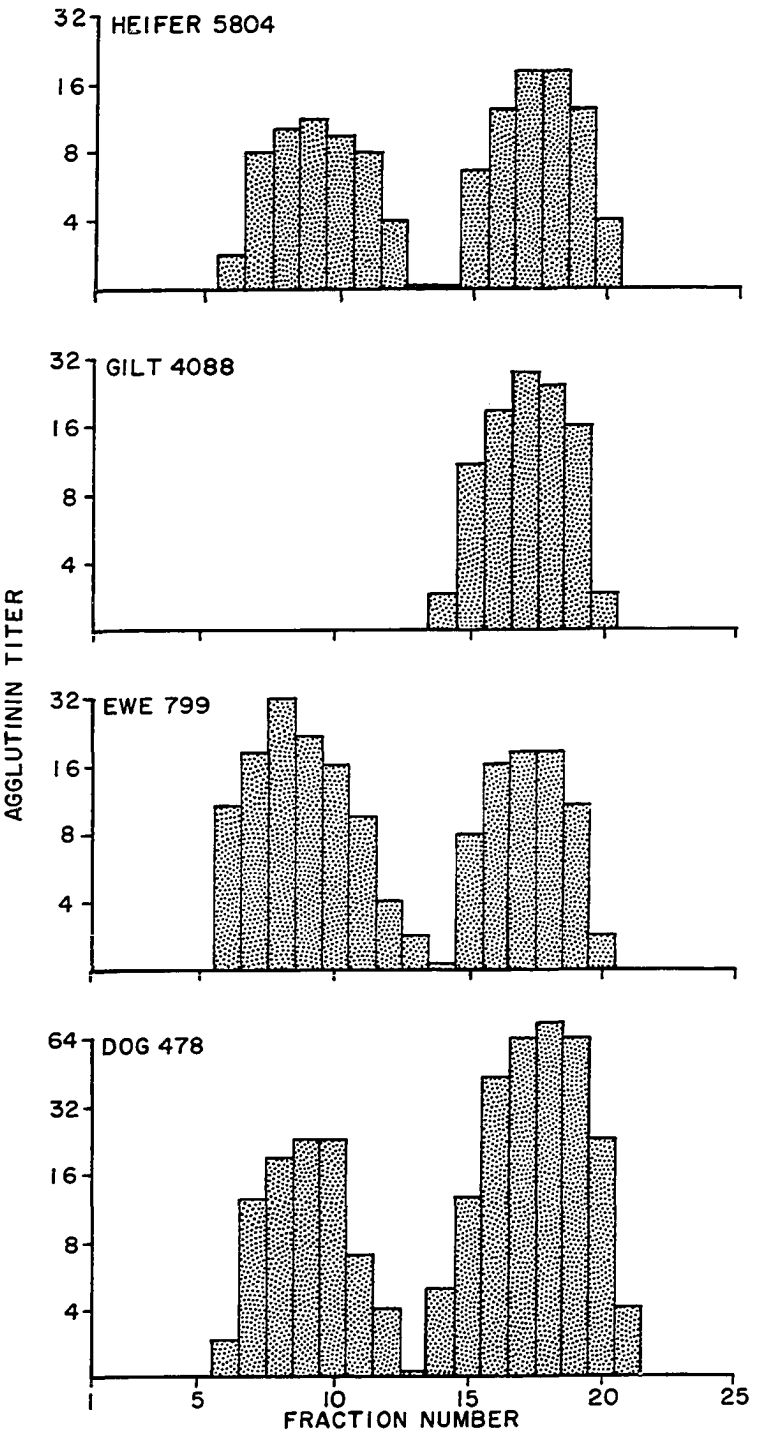
[c]No antibodies detected in any serum.

antibodies. Brucella abortus agglutinins were detected in serums from 4 swine during the course of the experiment, but these agglutinins were probably not related to exposure to Brucella.

Results of sucrose density gradient separation of Br. canis agglutinins in serums from swine, dogs, cattle and sheep correlated with results of ME tests (Figure 23). Serums which had both fast and slow sedimenting agglutinins were also test-positive with ME tests, whereas serums with only fast sedimenting agglutinins were test-negative with ME tests. Agglutination with slow sedimenting agglutinins proceeded at a much slower rate than with fast sedimenting agglutinins.

Bacteremia was never detected in any of the swine. Brucella canis was not isolated from gilts at parturition or from newborn pigs. However, Br. canis was recovered at necropsy from the 2 boars killed at 4 WPE, the 2 boars killed at 8 WPE, 1 gilt killed at 12 WPE, and 1 gilt killed at 13 WPE. Infection was not found in the 2 boars killed at 6 WPE or in the other 4 gilts, killed at 10 to 13 WPE. The source of Br. canis isolations was always the mandibular, suprapharyngeal, or parotid lymph nodes, with the latter two the most frequent sources. The infection was always unilateral and the numbers of organisms were usually less than 10 CFU/plate of medium.

Figure 23. The distribution of Br. canis agglutinins in serum-sucrose density gradients after ultracentrifugation. All serums shown were collected at 3 weeks postexposure. The fraction number indicates the depth in the gradient, from top to bottom, from which the 0.5 ml. fractions were taken


HEIFER 5804
GILT 4088
EWE 799
DOG 478
AGGLUTININ TITER
32
16
8
4
64
1
5
10
15
20
25
FRACTION NUMBER

Sheep

No clinical signs of disease were observed in sheep exposed to Br. canis. There were no distinguishable alterations in the quality of semen collected from rams during the course of the experiment. All pregnant ewes gave birth to normal lambs. Two ewes were nonpregnant at necropsy and there was no evidence that they had been pregnant. There were no gross lesions indicative of Brucella infection observed in any sheep at necropsy.

A moderate serologic response occurred as a result of exposure (Table 15). Pre-exposure serums of 8 sheep had agglutinin titers from 25 to 100. However, pre-exposure serums were all test-negative with Br. canis ME, CF, and GDP tests; with Br. abortus TA, ME, CF, and GDP tests; and with Br. ovis CF and GDP tests. After exposure to Br. canis the highest titers obtained with TA, ME, and CF tests were 560, 400, and 224, respectively. These titers were higher and more persistent than in cattle and swine, but still tended to recede after 5 to 6 WPE. Complement fixation test results with Br. ovis antigen were approximately equal to those obtained with Br. canis antigen. The Br. ovis GDP test was more effective than the Br. canis GDP test. Precipitins were detected in serums from 2 sheep with the former, but in serums of only one with the latter; also, lines of precipitation appeared sooner and were more visible with the Br. ovis antigen. No postexposure

Table 15. Geometric mean Br. canis antibody titers in serums of 12 sheep

Test	Weeks postexposure[a]										
	0	1	2	3	4	5	6	8	10	12	14
TA	55(8)[b]	58(11)	168	205	231	272	272	168	96	80	70
ME	--[c]	--	70(11)	97	158	206	162	114	59	43(7)	46(2)
CF	--	20(1)	16(8)	23(11)	31	44	32	34	22(6)	16(7)	40(1)
GDP[d]	--	--	(1)	(1)	(1)	--	(1)	(1)	(1)	(1)	--

[a]Only 10, 8, and 5 sheep remained in the experiment at 5, 10 and 14 weeks postexposure, respectively.

[b]Only titers of serums in which antibodies were detected used to calculate means; numbers in parentheses indicate number of animals in which serum antibodies were detected.

[c]No antibodies detected in any serum.

[d]GDP test results were obtained with a Br. ovis GDP test, which detected precipitins more frequently than the Br. canis GDP test; one sheep was GDP test-positive from 2 to 4 WPE and another from 6 to 12 WPE.

serum antibodies were detected with tests using Br. abortus antigens.

Antibodies were detected in serums from 3 of the 5 lambs born. The titers were 100, 70, and 28 in one; and 50, 25, and 14 in another, using Br. canis TA, ME, and CF tests, respectively. The TA test titer of the third (one of a pair of twins) was 25; but the serum was test-negative with ME and CF tests. Whey samples from the ewes were test-negative with all tests, even when the tests were conducted with whey dilutions as low as 1:2. The wheys were derived from milk collected from each lactating ewe on the day of parturition and at 3 days postpartum.

Bacteremia occurred in 7 sheep, but Br. canis was isolated from the tissues of only the 2 rams killed at 4 WPE (Table 16). The isolations at necropsy were from the parotid lymph nodes, a prescapular lymph node, and the spleen of ram 886 and from the parotid lymph nodes, a hepatic lymph node, and a superficial inguinal lymph node of ram 893. Relatively large numbers of organisms were isolated from the parotid lymph nodes, but few from the other sites. There was no bacteriologic evidence that Br. canis infection became established in reproductive systems of sheep as no organisms were recovered from semen of rams, from samples collected at parturition, from lambs, or from reproductive system tissues at necropsy.

Table 16. Bacteriologic evidence of *Brucella canis* infection in sheep

Sheep no.	Sex[a]	Necropsy (WPE)[b]	Bacteremia		Infected at necropsy
			Demonstrated	DPE[c]	
886	M	4	Yes	10,14,17	Yes
893	M	4	Yes	28	Yes
907	M	8	Yes	10,17	No
910	M	8	No	--	No
913	M	12	Yes	10,24	No
962	M	12	No	--	No
513	F	15	No	--	No
516	F(NP)	14	Yes	14,17,21	No
799	F	15	Yes	14	No
800	F	14	No	--	No
801	F(NP)	13	Yes	10,21	No
818	F	15	No	--	No

[a]M = male; F = pregnant female; F(NP) = nonpregnant female.

[b]WPE = weeks postexposure.

[c]Days postexposure when blood that contained *Br. canis* was collected.

Comparative Virulence of *Brucella canis*

Guinea pigs

The development of disease in guinea pigs inoculated with *Br. canis* RM6-66 and in those inoculated with *Br. suis*, type 3, strain 688 differed considerably (Table 17). *Brucella canis* was isolated only from spleens, while *Br. suis* was isolated from various tissues and from larger numbers of guinea pigs. The numbers of *Br. suis* in spleens was usually much greater than the numbers of *Br. canis* in spleens. The gross lesions due to *Br. canis* were very mild enlargement of spleens, whereas lesions due to *Br. suis* were marked enlargement of spleens and lymph nodes and multiple necrotic foci in spleens, livers, and epididymides.

In this experiment, *Bordetella bronchiseptica* was recovered from the lungs of 13 of 48 *Brucella*-infected guinea pigs and from the lungs of 3 of 12 uninoculated control guinea pigs. Investigation of the distribution pattern of these infections led to the conclusion that *Bordetella* infection was present in one or more of the guinea pigs before the experiment was initiated. There was no evidence, however, that *Bordetella* infection interfered with the development of *Brucella* infection in guinea pigs. Microscopic tissue changes caused by *Br. canis* and *Br. suis* infections were not evaluated because of the foregoing complication and because technical problems in fixation and sectioning tissues caused many tissues to be unsuitable for histologic examination.

Table 17. Development and distribution of Br. canis and Br. suis infections in guinea pigs

No. of pigs	DPI at necropsy[a]	No. of Brucella isolated[b]	No. of guinea pigs affected: Isolation of organisms from: Spleen	Liver	Lungs	Epididymis or uterus	Serum agglutinins	Gross lesions	Bordetella infection
Br. canis infection									
4	3	3.7	2	0	0	0	0	0	1
4	7	3.6	1	0	0	0	0	0	2
4	10	3.8	3	0	0	0	2	2	1
4	14	3.6	1	0	0	0	1	0	1
4	21	3.5	3	0	0	0	3	1	2
4	28	2.9	3	0	0	0	3	0	2
Br. suis infection									
4	3	3.8	2	1	0	0	0	0	0
4	7	4.3	3	3	0	0	0	2	0
4	10	6.3	3	3	1	3	3	3	1
4	14	5.7	4	4	1	0	4	2	0
4	21	4.7	4	3	0	0	2	3	2
4	28	5.9	4	4	2	2	4	4	1

[a]DPI = days postinoculation.

[b]Mean of the logarithm of colony forming units/infected spleen.

In the second guinea pig experiment, Br. canis was significantly less virulent than the other Brucella tested (Table 18). In fact, Br. canis infection failed to persist as long as 42 days after inoculation, although a few guinea pigs still had serum agglutinins at that time. Comparison of the indices of infection indicated persistent or progressive infection (stable or increasing index) with Br. suis and recovery from infection (marked decrease in index) with Br. abortus strain 19 and Br. canis. The guinea pig virulence of the 4 strains was characterized as: high virulence - Br. suis 3b; moderate virulence - Br. suis 688; low virulence - Br. abortus strain 19; and very low virulence - Br. canis.

Macrophage cultures

The ability of normal guinea pig peritoneal macrophages to ingest and apparently destroy Br. canis and Br. suis differed markedly between the 2 organisms (Table 19). Viability counts of intracellular Brucella indicated that Br. canis organisms were phagocytized more readily than Br. suis, but subsequent intracellular multiplication of Br. canis was quite limited or nonexistent. The apparent capability of the macrophages to ingest and destroy Br. canis was approximately 1,000 times that with Br. suis. Thus, the response of guinea pig macrophages to the 2 organisms correlated with the guinea pig virulence.

Table 18. Comparative virulence of 4 strains of *Brucella* for guinea pigs

Inoculum	No. infected/ no. inoculated		Index of infection[a]	
	21 DPI[b]	42 DPI	21 DPI	42 DPI
Br. suis, strain 3b	10/10	10/10	18.1	18.3
Br. suis, strain 688	10/10	10/10	12.0	13.2
Br. abortus, strain 19	10/10	5/10	7.4	3.9
Br. canis, strain RM6-66	5/10	0/10	3.0	0.6

[a]Cumulative total of points designated for severity of disease divided by number of guinea pigs in the group.

[b]DPI = days postinoculation.

Table 19. Response of guinea pig peritoneal macrophages to *Br. canis* and *Br. suis*

Postinoculation interval (hrs.)	Mean no. of intracellular organisms[a]	
	Br. canis	*Br. suis*
0[b]	6.81	6.88
2	5.52	4.52
21	4.60	3.70
44	2.19	4.26

[a]Expressed as mean of the logarithm of colony forming units/tube; derived from duplicate tubes from each of 2 experiments with identical design.

[b]Number of organisms inoculated into each tube.

Microscopic enumeration of the numbers of intracellular Brucella in stained cell cultures correlated with the viability count data, but the latter were considered far more accurate enumerations. Neither Br. canis nor Br. suis had any distinguishable toxic effect on macrophage cultures. The proportion of cells permeable to Trypan Blue in infected cultures varied from 7% to 18%, while the proportion in noninfected control cultures was 16% to 23%.

Immunofluorescent staining of infected macrophage cultures provided comparisons similar to the other methods, but counting of intracellular organisms was impossible because diffuse staining, probably due to presence of soluble antigen, was observed in some cells. Also, immunofluorescent staining was far less sensitive for detection of intracellular Brucella than the other methods. No ultrastructural changes were observed in macrophages from infected cultures (Figure 24).

Morphologic, Physiologic, and Serologic Characteristics of Brucella canis

The colonial appearance of Br. canis was typical of the genus Brucella, except that all colonies were mucoid, thereby differing from primary isolates of previously-recognized, naturally-occurring Brucella. The Br. canis cells were small, nonmotile, Gram-negative coccobacilli, usually arranged singly. However, Br. canis stained more intensely with the Gram stain than Brucella usually do. Also, Br. canis reduced nitrates and produced oxidase, catalase and urease, but did not ferment

Figure 24. Electron micrograph of part of a guinea pig peritoneal macrophage from a culture exposed to Br. canis organisms. There is no discernible alteration in the ultrastructure of the macrophage. There is an extracellular Br. canis organism at the lower left. Lead citrate and uranyl acetate stain, X95,800

carbohydrates in usual mediums, liquefy gelatin, utilize citrate, grow on MacConkey agar, hemolyze blood, or alter litmus milk--all characteristics that are typical of the genus Brucella.

Brucella canis did not require added CO_2 for growth. In fact, growth was usually inhibited slightly by added CO_2. It had nigh catalase activity (1.5 to 2.5 milliequivalents H_2O_2 decomposed/10^9 cells) and rapidly hydrolyzed urea. Since these characteristics were typical of Br. suis, the preliminary observation was that it might be difficult to distinguish Br. canis from nonsmooth Br. suis, produced by laboratory manipulation.

Results of further tests, useful for division of Br. suis into biotypes, indicated that Br. canis could be distinguished from recognized biotypes of Br. suis (Table 20). Only the median results are presented in Table 20, but variations among cultures of the same biotype were slight. All test mediums were inoculated with rather precise numbers of organisms (about 1 X 10^8/plate). The pattern of dye bacteriostasis of Br. canis was similar to, but distinguishable from, Br. suis, type 2 and the Huddleson-M strain. It was also distinguishable from the latter on the basis of H_2S production, and from the former on the basis of colony morphology and serotype.

The characteristics of isolates of Br. canis and Br. suis 688 from experimental animals were identical to the original cultures. The Br. canis cultures were 100% mucoid, of the

Table 20. Differential features of *Br. canis* and *Br. suis* cultures

Species	Biotype	No. of strains	No. of cultures	Colony morphology	*Brucella* phage lysis (10^4 RTD)	Serotype[a]	H_2S production[b]
Br. suis	1	39	53	Smooth	±	A	++++
Br. suis	2	5	7	Smooth	-	A	±
Br. suis	3	20	36	Smooth	-	A	±
Br. suis[c]	1	1	2	Rough-mucoid	-	R	++++
Br. canis	-	1	41	Mucoid	-	R	-

[a]Serotype determined by agglutination with monospecific *Br. abortus* serum (A), monospecific *Br. melitensis* serum (M), or anti-rough *Brucella* serum (R).

[b]Results expressed as: - to ++++, depending on the degree of H_2S production and growth on dye mediums.

[c]This strain was identified as Huddleson-M, a laboratory-produced mucoid *Br. suis* that was used as an experimental vaccine at one time (36).

Table 20. (Continued)

Species	Biotype	No. of strains	No. of cultures	Growth on tryptose agar containing dyes (mg./liter)[b]								
				Thionin					Basic fuchsin			Safranin O
				100	40	20	10	5	20	10	5	200
Br. suis	1	39	53	++	++++	++++	++++	++++	-	-	-	-
Br. suis	2	5	7	-	-	-	++	++++	-	-	-	-
Br. suis	3	20	36	-	+	++++	++++	++++	+	++++	++++	++
Br. suis[c]	1	1	2	-	++	++++	++++	++++	-	-	+++	-
Br. canis	-	1	41	-	-	+	++++	++++	-	-	+++	-

M-type described by Carmichael (15), as near as could be determined. All Br. suis 688 cultures were characterized as 100% smooth. In addition, the guinea pig virulence of a 4th canine passage culture of Br. suis 688 was indistinguishable from that of the original culture isolated from swine, and the guinea pig virulence of 4th canine passage Br. canis was the same as the original culture.

Tests for serologic cross-reactions among Brucella and other selected bacteria indicated that Br. canis was closely related to nonsmooth Brucella (Table 21). Major, reciprocal cross-reactions between heterologous genera were rare. Usually, smooth Brucella antigens reacted strongly only with smooth Brucella antiserums, and nonsmooth Brucella only among themselves. However, Br. suis 1330 (smooth) cross-reacted with both smooth and nonsmooth Brucella, and was thereby qualitatively different than the other smooth Brucella. The antigens prepared by autoclaving cell suspensions of Br. canis and Br. ovis (naturally-occurring, nonsmooth) were qualitatively different than antigens prepared in the same manner from the laboratory-produced, rough Brucella (Br. abortus 45/20 and Br. suis PR-1). Antigens prepared from the latter contained no detectable precipitinogens.

Two factors tended to confuse interpretation of results of the serologic trials. First, not all antiserums had equivalent titers with their homologous antigens. In particular, the Br. ovis antiserum had markedly less antibody content than

Table 21. Serologic cross-reactions among *Brucella* and other genera[a]

Antigen		Antiserum against antigen no.:							
No.	Identity	1	2	3	4	5	6	7	8
1	*Br. canis*, RM6-66	ACP	A--	ACP	ACP	acp	---	a--	---
2	*Br. ovis*, 692	ACP	ACP	ACP	ACP	acp	---	a--	---
3	*Br. suis*, PR-1	AC-	A--	AC-	AC-	Ac-	a--	ac-	---
4	*Br. abortus*, 45/20	AC-	a--	AC-	AC-	AC-	Ac-	A--	a--
5	*Br. suis*, 1330	acP	---	---	AC-	ACP	ACP	ACP	ACP
6	*Br. abortus*, 544	ac-	---	---	AcP	ACp	ACP	ACP	ACP
7	*Br. melitensis*, 16M	ac-	---	-c-	ACP	ACP	ACP	ACP	ACP
8	*Br. neotomae*, 649	acp	---	-c-	ACP	Acp	ACP	ACP	ACP
9	*B. bronchiseptica*, 546	---	---	-c-	---	---	---	---	---
10	*B. bronchiseptica*, 711	---	---	-cp	---	---	---	---	---
11	*Actinobacillus*	---	-c-	a--	a--	---	---	ac-	---
12	*Alcaligenes*	-c-	---	-c-	a--	---	---	---	---
13	*Moraxella*	---	---	-c-	-c-	---	---	---	---
14	*Pasteurella*	a--	a--	---	---	---	---	a--	---

[a] A = agglutination, C = complement fixation, P = precipitation; capital letters = strong reaction (no greater than 8-fold difference from homologous titer), lower case letters = weak reaction (16-fold to 64-fold difference from homologous); --- = no reaction.

Table 21. (Continued)

Antigen		Antiserum against antigen no.:					
No.	Identity	9	10	11	12	13	14
1	Br. canis, RM6-66	a--	---	a--	---	---	---
2	Br. ovis, 692	a--	a--	ac-	---	ac-	---
3	Br. suis, PR-1	a--	a--	---	---	---	---
4	Br. abortus, 45/20	---	---	ac-	-c-	---	---
5	Br. suis, 1330	---	--p	ac-	-c-	---	---
6	Br. abortus, 544	---	--p	acp	---	---	---
7	Br. melitensis, 16M	---	---	ac-	---	---	---
8	Br. neotomae, 649	---	---	ac-	---	---	---
9	B. bronchiseptica, 546	ACP	ACP	acp	---	---	---
10	B. bronchiseptica, 711	ACP	ACP	acp	---	---	---
11	Actinobacillus	---	---	ACP	---	---	---
12	Alcaligenes	---	---	AC-	AC-	---	---
13	Moraxella	---	--p	---	---	ACP	--p
14	Pasteurella	---	--p	---	a--	---	ACP

most others and a repeated attempt to produce satisfactory antiserum was unsuccessful. The homologous endpoint agglutinin titers of antiserums were 1600 to 6400, except for Br. ovis, Moraxella, and Pasteurella serums which were 800, 400, and 400, respectively. The CF titers were 320 to 1280, except for Br. ovis and Moraxella which were both 80. There were seldom more than 1 or 2 lines of precipitation formed with any antigen-antiserum combination in the gel diffusion tests. Secondly, the Br. abortus 45/20 (originally obtained from Great Britain in 1944) appears to be a pure rough strain upon cultural examination, but multiple injections or injection of large doses of live organisms into animals invariably results in proliferation of smooth forms in vivo. Consequently, the Br. abortus 45/20 antiserum contained antibodies for both smooth and nonsmooth Brucella.

DISCUSSION

A major objective of this research was to compare Br. canis and Br. suis infections in dogs. To provide the necessary latitude, studies of clinical, serologic, bacteriologic, and pathologic aspects of the 2 infections were conducted. Review of the results obtained revealed definite differences. Also, comparisons with the known pathogenesis of brucellosis in other animals were helpful in achieving perspective. There is a distinct possibility of error, however, unless such comparisons are limited to one animal species infected with different Brucella species or to different animal species infected with one Brucella species.

Under the conditions of the experiments, the clinical aspects of Br. canis and Br. suis infections differed. Brucella canis caused no interference with reproduction but Br. suis regularly caused reproductive failures. It was obvious that Br. suis was capable of producing a pathologic effect on genital organs much more rapidly than Br. canis.

Pyrexia, attributable to Brucella infection, did not occur. Suggestions (17, 79) that lack of pyrexia may be due to lack of endotoxin in Br. canis organisms have little merit. Brucella suis infection also failed to cause fever in this study. Moreover, pyrexia associated with brucellosis is a common finding only in man; it is seldom demonstrable during the course of brucellosis in domestic animals.

Clinically apparent lymphadenopathy was found only after 2 to 3 months postexposure and only in dogs exposed to Br. canis. To describe palpable lymph node enlargement in any dog during the early postexposure period would have been imagery.

Bacteriologic and serologic data obtained from Br. canis-infected dogs indicated a high, persistent multiplication of organisms in vivo and, therefore, persistent and massive antigenic stimulation. The magnitude and persistence of bacteremia were far greater than that heretofore observed in brucellosis of any animal. Four-month-old dogs and neonatal pups were apparently as susceptible to Br. canis as sexually mature dogs.

Conversely, data from Br. suis-infected dogs indicated a rather brief bacteremic phase, with primary localization of organisms in the genital tract. Organisms also persisted in the lymphatic system. It appeared that most Br. suis-infected adult dogs were in a recovery phase by 4 to 6 weeks postexposure. Apparently, the 4-month-old dogs entered the recovery phase sooner than adults because: the bacteremic period was very brief, antibody titers rapidly receded after the initial rise, organisms were recovered only from the lymphatic system at necropsy, and there were no histopathologic changes.

Despite the much higher multiplicity of Br. canis in many tissues, histopathologic changes in reproductive systems were induced very slowly by Br. canis, but very rapidly by Br. suis. In fact, genital tract lesions due to Br. canis in female dogs occurred too seldom to compare them.

The course of Br. suis infection, and particularly the development of placentitis, in pregnant bitches can be postulated from the data obtained under the conditions of these experiments. After exposure the organisms are ingested by phagocytes at or near mucosal surfaces and carried to regional lymph nodes through lymph channels. The Br. suis organisms multiply and escape, mostly within phagocytes. A state of brucellemia occurs, usually within a week after exposure, thereby disseminating organisms throughout the body.

Organisms invading the uterus localize in the uterine glands and lamina propria and stimulate an inflammatory response. The inflammation spreads into the myometrium, supraglandular connective tissue, and the spongy layer of the placenta. Brucella suis organisms congregate and multiply at the junction of the spongy layer and placental labyrinth. The organisms are engulfed by trophoblastic epithelium of the terminal chorionic villi, but also occur as extracellular colonies. This area evidently provides an ideal environment for multiplication of Br. suis due to a rich source of nutrients, lack of inhibitory mechanisms, or both. Extensive necrosis of glandular tissue of the spongy layer and terminal chorionic villi, as well as suppuration, results. Extensive tissue destruction in this area is especially critical because the placenta separates through the spongy layer at normal parturition. Premature and extensive necrosis of the spongy

layer leads to premature separation of the placenta and is, therefore, the basic cause of the abortion.

Abortion ensues after placentas begin to detach and are free in the uterine lumen. This occurred about 4 weeks after exposure in this study. Since the pathologic changes in different placentas of the same uterus are rarely at the same stage at any given time, nonviable fetuses and fetuses in all stages of viability will be aborted. Retained placentas are not a feature of canine brucellosis because the zonary, deciduate placenta of the dog does not lend itself to such a phenomenon. The aftermath of placentitis and abortion due to Br. suis would likely be temporary or permanent infertility because of the severe uterine alterations.

Multiple focal degenerative changes occur in chorionic villi within the green border and in the lamellae of the placental labyrinth, but are probably secondary in importance to the previously-mentioned pathologic alterations. The changes in the lamellae are a fibrinoid degeneration of the maternal capillaries and the adjacent syntrophoblast. This is the lesion that is apparent grossly as a yellowish discoloration within the placental labyrinth. The cause of the alteration is unclear, but could possibly be attributed to an antigen-antibody reaction at the site or to the effects of an unidentified toxin. Undoubtedly, the result of the lesion is decreased efficiency of the placental function.

Gross pathologic alterations in the placenta precede visible effects on the fetus. Stunting of the fetus, however, usually takes place before separation of the placenta occurs. Antemortem autolytic changes occur in the fetus and are characterized mainly by hemorrhagic effusions. Death of the fetus is usually preceded by detachment of the placenta. After detachment of the placenta and fetal death, autolysis of the deciduate placenta and fetus progresses rapidly.

Although enormous numbers of Br. suis organisms are present in placental fluids and fetuses, the fetus apparently does not respond to the infection. The only changes that occur in the fetuses are autolytic changes. Dillman (27) has outlined the sequential alterations that occur during fetal autolysis. Lack of fetal lesions in canine brucellosis is similar to the usual findings in brucellosis of other species. A notable exception is experimental Br. ovis infection in sheep. In fetal lambs infected with Br. ovis, proliferation and infiltration of lymphoreticular cells occur in various organs, and the occurrence is dependent on the maturity of the fetal immunologic system (68, 69).

Placentitis did not occur in Br. canis-infected bitches under the conditions of these experiments. Therefore, no direct comparisons with the development of placentitis postulated for Br. suis infection are possible. Carmichael and Kenney (16) stated that the basic placental lesion due to

Br. canis was focal coagulation necrosis of chorionic villi and that trophoblastic epithelial cells often contained numerous bacteria, but they did not elaborate further. It is likely that, except for the length of time required for development, placentitis due to Br. canis and to Br. suis would be similar. Perhaps the comparison of placentitis in dogs caused by Br. suis and Br. canis is analogous to the comparison between experimental Br. melitensis and Br. ovis infections in sheep. Molello et al. (56, 57) found that Br. melitensis rapidly caused abortion, whereas a lengthy incubation period was necessary before Br. ovis infection would induce abortion; also, the locations of placental alterations were different.

Microscopic uterine lesions due to Br. canis were found in the dog exposed at 10 days of gestation, but not in dogs exposed later in gestation. Perhaps the dog exposed at 10 days of gestation would have developed gross placentitis if she had been retained until parturition. The reports (13, 16, 17) that Br. canis causes early abortions is plausible, but only if uterine infection was well-established at the time of breeding. It appears that Br. canis could induce a high rate of abortions at 7 to 9 weeks of gestation when exposure occurs at or very shortly after breeding.

The histopathologic changes induced by Br. canis in the one male dog were very similar to those described by Moore and Kakuk (62). A report (17) has intimated that the genital lesions in male dogs are dissimilar to brucellosis in bulls and

boars because of lack of orchitis in dogs, and further, that the epididymitis in dogs is similar to Br. ovis infection in rams. On the contrary, the genital lesions in male dogs are very comparable to those caused by Br. suis in boars and Br. abortus in bulls (23, 48). The frequency of orchitis is usually overstated in reviews on brucellosis. Although orchitis may occasionally develop as a result of Brucella infection, it is nearly always preceded by chronic epididymitis. Epididymitis in rams, due to Br. ovis, is unlike the epididymitis described for Br. canis infection in dogs. In rams the basic pathologic change is development of spermatic granulomas (41).

Changes in lymph nodes of Br. canis-infected and Br. suis-infected dogs were not clearly distinguishable during the first 6 WPE. Progressive lymphoid hyperplasia was the outstanding alteration in nodes of Br. canis-infected dogs. Marked plasmacytosis was frequently observed. The characteristic lesion in lymph nodes of cattle infected with Br. abortus and swine infected with Br. suis is reticuloendothelial hyperplasia (23, 71). Therefore, the reaction of the lymphatic system of dogs to Brucella infection is probably a nonspecific response characteristic of canines.

Alternatively, the lymphatic system response to Br. canis might be contrasted to the findings of Margolis et al. (49) in experiments where dogs were repeatedly given massive intravenous doses of Br. suis in an attempt to produce a Hodgkin's-

like disease. They reported a pronounced reaction involving sinus endothelium and reticulum cells. There was development of focal epithelioid granulomas in lymphatic cords and formation of great masses of large mononuclear wandering cells, without enlargement of the nodes. The end result was replacement, rather than proliferation, of germinal centers. Their findings are not comparable to Br. suis infection because of the exposure method. They may be comparable to Br. canis infection, however, where the high multiplicity of organisms might be analogous to the assault produced by repeated parenteral injections.

Evidence of extramedullary hematopoiesis, particularly in spleens, might have been caused by the withdrawal of 2 to 4% of the blood volume of each dog weekly. In addition, spontaneous hemolysis occurred frequently in blood samples collected from a few individual infected dogs. Hematocrit determinations and differential leucocyte counts were conducted sporadically during the experiments, but the results were all within normal ranges and did not reflect anemia or leucocytosis.

The hepatic lesions in Br. canis and Br. suis infection differed. The lesions in Br. canis-infected dogs consisted of focal accumulations of lymphocytes and macrophages in the sinusoids and were found only in dogs infected for about 6 weeks or longer. Brucella suis infection produced necrotic foci and the lesions were observed in dogs killed as early as 24 days postexposure.

Necrotizing arteritis has been described for Br. canis infection (17), but it was not observed in this study. Although sections of the central nervous system were not examined microscopically in this study, there was no bacteriologic, gross pathologic, or clinical evidence of meningitis, such as that reported (17).

In this study, all aspects of Br. suis infection in female dogs appeared to be very similar to Br. suis infection in swine (23, 24, 37, 46). In addition, there were no findings to suggest significant differences between Br. suis and Br. abortus infections in dogs (65, 66, 67, 90).

I cannot agree that dogs are relatively resistant to infection with Br. suis (41, 65). After exposure of dogs to moderate doses of Br. suis by a natural route bacteremia rapidly developed, there was dissemination of organisms throughout the body, infection persisted, marked pathologic changes occurred in tissues, pregnant dogs aborted, and organisms were recovered from the vaginal canal, milk, and respiratory passages. One could confidently speculate that the only reason enzootics of canine Br. suis infection have not occurred is because the infection has never been introduced onto premises where dogs kept for breeding purposes are closely congregated.

The cautions expressed by Moore and Gupta (60) about other causes for signs similar to those of Br. canis infection should be heeded. Two dogs in this study were accidentally

infected with Strep. uberis and the suppurative pyelonephritis, suppurative epididymitis, and pyrexia that occurred in one was probably caused by the streptococcal infection. The source of the streptococci was unknown. However, the 2 dogs were in a room adjoining one with cattle for 10 weeks, and the same personnel cared for both groups of animals.

The lymph nodes and spleens of nonexposed control dogs in this study contained lesions suggestive of Brucella infection. Such findings were not unique to this study. Wipf et al. (90) had a similar experience in the examination of lymph nodes for effects of canine Br. abortus infection.

Parasitic granulomas were present in the renal cortex of the majority of dogs in this study. Analogous lesions have, at times, been ascribed to Brucella infection (65, 90). Similar lesions were also observed by Moore and Kakuk (62) during their study of Br. canis infections. They, however, wisely suggested that the lesions could be due to ascarid larvae migration.

The foregoing complications might have been avoided if use of germ-free dogs could have been justified; certainly, greater histologic contrast between controls and principals should be demonstrable. Nevertheless, the dogs used were obtained from reputable kennels and were undoubtedly representative of kennel-raised canine populations.

Immunofluorescent techniques and tinctorial stains for bacteria were helpful in evaluating placental changes due to Br. suis infection, but neither method was particularly useful

in evaluating other tissues. The use of fluorescent antibody for localization of Brucella in tissues has some inherent handicaps. First, as previous studies indicated (55, 74, 76), coating of antigen in vivo by circulating antibody can prevent later attachment of the labeled antibody. Methods developed by Prichard (74) to overcome this were probably not highly effective with tissue sections, even though they were quite effective with pure cultures of antiserum-treated Brucella.

Secondly, not all organisms, even in pure culture, stain brightly. Difficulties in achieving FA reagents that stained Br. canis satisfactorily were not unexpected. Brucella ovis, antigenically very similar to Br. canis, does not stain with specific fluorescein-labeled antibody (55). Furthermore, serologic evidence in this study suggested that the mechanism of antigen-antibody reaction with Br. canis may be different than with smooth Brucella.

Finally, visible FA staining is dependent on the number of organisms present. When methods for concentrating the organisms are incorporated in the procedure, as few as 1×10^3 Brucella/ml. of bovine milk (74) and 4.4×10^6 Brucella/ml. of blood (76) can be detected. Prichard (74) could detect organisms in sections from guinea pig spleens containing about 2×10^4 organisms/Gm., if 12 to 16 sections from each spleen were examined. Assuming uniform distribution of organisms and 100% efficacy of the staining reaction, there should be one visible organism per high power field in sections cut from

tissues containing 1 X 10^7 organisms per gram. Therefore, concentration of organisms, either by chance or design, is usually necessary for useful immunofluorescence of Brucella in tissues.

Failure of electron microscopy to contribute materially to the data can be attributed to the limited amount of tissue that was examined and to errors in selection of tissues. Reviews of literature and previous experience indicated that medullary portions of lymph nodes and lamellae of the placental labyrinths would be fruitful areas to examine. Later observations revealed the error in judgment.

Results of serologic procedures conducted indicated that 2 types of agglutinins, presumably 7S and 19S, were produced by dogs, cattle, and sheep in response to Br. canis exposure. Swine produced only 19S agglutinins. It appeared that the 19S antibodies in serums from infected dogs and sheep fixed complement, while those from swine and cattle did not. The 19S agglutinins in sucrose density gradient fractions rapidly agglutinated Br. canis antigen, while the 7S agglutinins did not--a situation in reverse to that previously observed with agglutinins to smooth Brucella.

The concept that incubation of agglutination tests at 50 ± 2 C. is essential for satisfactory results (13, 15, 60, 72) is contrary to my findings. Possibly the disagreement arises from definitions of "satisfactory." I believe the two major criteria for a satisfactory agglutination test are

readability and reproducibility; the nature of the agglomerate need not be esthetically desirable. The agglomerate in the test used was a mucoid mass, further intensified by addition of gelatin stabilizer to the diluent; however, test results were readily determined by observation of clearing and clumping. Continual use of positive and negative control serums, as well as repeated tests on many serums, failed to indicate lack of reproducibility. Incubation of tests at 50 C. for 24 hours always achieved the same results as incubation at 37 C. for 48 hours, but it was not possible to use the mercaptoethanol test at 50 C.

It would seem absurd to accept the agglutination test as a standard method for diagnosis of Br. canis infections without thorough evaluation of other methods. If agglutinin titers greater than 100, complement fixing titers greater than 10, and positive GDP tests can be arbitrarily accepted as indicative of infection, the CF and GDP tests were superior to the TA test in this study. They (CF and GDP) were more sensitive in detecting early infection in dogs and also considerably more accurate in assessing the course of infection in other animals. The agglutination tests seemed to be prone to detect heterospecific agglutinins (due to causes other than Brucella) frequently in this study, and similar observations have been reported (13, 72). However, regardless of the comparative merits of serologic tests, Br. canis infection in dogs appears to provide an opportunity for use of more conclusive diagnostic

methods. Because of the ease and frequency by which Br. canis organisms can be isolated from blood, tissues, and exudates, bacteriologic examinations should be the method of choice and serologic tests used as a supplemental procedure. Other recommendations along this line appear to be well-founded (60, 61).

The conjunctival exposure method used in this study was considered appropriate. When one swabs the conjunctival sac, nasal cavity, and pharynx 30 minutes to 1 hour after conjunctival exposure, about equal numbers of organisms can be recovered from each of the 3 locations. Therefore, the conjunctival method actually provides almost simultaneous oral, ocular, and intranasal exposure, and it does not by-pass possible defense mechanisms that parenteral injections would. The exposure doses for cattle, swine, and sheep were increased over that normally necessary because it was assumed that these animals would not be a definitive host for Br. canis and that there should be some allowance for susceptibility to massive exposure. Positive and negative control animals were not used in the study because cattle, swine, and sheep from the same source had previously been susceptible to Br. abortus, Br. suis, and Br. ovis, respectively, and because nonexposed controls would have been of no value in this type of study. The results of trials on the pathogenecity of Br. canis for cattle, swine, and sheep do not agree with those reported by Pickerill (72), although the ultimate conclusions are probably the same.

There was no bacteriologic evidence that cattle exposed to Br. canis became infected. However, a transient infection that stimulated the production of antibodies must have occurred. The susceptibility of cattle to Br. canis may be similar to their susceptibility to Br. suis. Although Br. suis, type 1 (but not other biotypes) has been found in mammary glands and milk of cattle on rare occasions, experimental infection is very difficult to produce except by intramammary exposure (48, 88).

Swine developed a localized infection in lymph nodes of the head, but there was no evidence that infection spread beyond this area. The infection could not have been diagnosed serologically. This is comparable to the susceptibility of swine to Br. abortus. This organism has been encountered in mandibular lymph nodes of swine (52), but has never been known to spread from swine to swine or cause abortion in swine (54).

There was irrefutable evidence that Br. canis caused systemic infection in sheep, as evidenced by bacteremia and the distribution of organisms isolated at necropsy. However, the infection was apparently quite self-limiting and had no discernible effect on reproduction. It cannot, however, be stated that Br. canis organisms or antigens did not invade the uterus. Wheys from ewes did not contain Br. canis antibodies, but serums from their lambs did. This was contrary to the findings in neonatal calves, pigs, and pups. The antibodies in lambs were probably produced by the fetus in utero.

Osburn and Hoskins (68) have demonstrated that fetal lambs can produce I_gM and I_gG immunoglobulins to Br. ovis as early as 98 days of gestation. Pregnancy apparently had no effect on the susceptibility of sheep to Br. canis infection. It was not surprising that sheep were found to be more susceptible than cattle or swine because sheep are relatively susceptible to a variety of Brucella species (54).

It was unusual that Br. canis seemed to have a predilection for parotid lymph nodes of swine and sheep. Usually Brucella organisms are found more frequently in mandibular or suprapharyngeal nodes than in parotid nodes (25, 44).

From data obtained in this study and review of the literature it appears that the host range of Br. canis is primarily limited to canines. The disease is widespread among kennels and it can cause serious economic loss where dogs are closely congregated (14, 72). To the author's knowledge, the disease has not yet been positively diagnosed in Iowa. There has been no evidence that progressive infection with animal-to-animal transmission would result if, by chance, domestic livestock were exposed to infected dogs. Whether the disease will ever become a significant problem in family pets, even without any effort to control it, is questionable. Moreover, the present rate of human infections with Br. canis--about 2 cases/year--hardly constitutes a public health problem. Therefore, responsibility for control of the disease should rest with dog

breeders and those segments of veterinary medicine that serve them.

Findings in this study are not in agreement with reports that a variety of lesions in guinea pigs are caused by Br. canis (13, 15). On the contrary, the organism was found to be of very low virulence for guinea pigs. Determinations of its intracellular multiplication in guinea pig peritoneal macrophages correlated with the results in the live animal. This suggests that the host range of a given Brucella species is dependent on the ability of the host macrophages to phagocytize the organisms and limit their intracellular multiplication. No ultrastructural changes were observed in infected macrophages in culture, and none were expected. In extensive studies, Brucella abortus organisms also failed to induce specific ultrastructural changes in phagocytes (92).

The characteristics of Br. canis strain RM6-66 were studied extensively in this research. Colony selection was avoided and the number of culture transfers was limited as much as possible, in an effort to avoid production of laboratory artefacts. The characteristics of the cultures did not vary significantly from one to the other, and they were not altered by animal passage. Therefore, the mucoid phase of this agent is apparently a stable, pathogenic form.

The general characteristics of Br. canis were similar to those of Br. suis. However, when critically examined, it was unlike any other Brucella. It has some distinctive

characteristics, particularly its colonial morphology and pattern of biochemical reactions, that could not be duplicated with nonsmooth Brucella. These findings agree, in general, with reports on similar studies of strain RM6-66 (15, 40, 53), but there were minor disagreements: (a) Br. canis is not rough (40, but mucoid; and (b) the biochemical reactions are more like Br. suis, type 2 than Br. suis, type 3 (40, 53). The greatest weakness of cultural investigations herein was that only one strain of Br. canis was examined. It is possible that other strains may vary slightly from strain RM6-66. Meyer (53) found that different strains of Br. canis differed somewhat in their oxidative metabolic patterns.

It is expected that the Subcommittee on Taxonomy of the Genus Brucella, International Committee on Nomenclature of Bacteria will recommend a taxonomic position for the canine abortion organism (17, 53). The Subcommittee met in August, 1970, and recommended that it be included in the genus Brucella, but species and biotype designation was held in abeyance until more strains could be studied.[1] There have been 2 different proposals for species designation: Br. canis (15, 40, 51, 59) and Br. suis, biotype 5 (53). Although the canine organisms are quite similar to Br. suis, the disease

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[1]Meyer, M. E., Davis, California. Proceedings of Subcommittee on Taxonomy of Brucellae meeting. Private communication. September, 1970.

caused by them seems to be a distinct entity, apart from Br. suis infections. Even though taxonomists are often reluctant to consider epizootiologic evidence in resolving nomenclature disputes, the name Br. canis would provide a definite identity for this discrete organism that causes a distinct disease, but burying it within Br. suis would not. Moreover, one of the few practical applications for bacterial taxonomy is its usefulness in epizootiology. Since the designation Br. canis has already been widely accepted, it may continue to be the predominant terminology, regardless of decisions rendered by the Subcommittee on Taxonomy.

SUMMARY

Brucella canis and *Br. suis* infections in dogs were compared. The 17 Beagles utilized in the experiments included pregnant females and prepuberal dogs. Clinical, serologic, bacteriologic, and pathologic aspects of the infections were investigated.

Dogs exposed to *Br. suis* during pregnancy developed gross placentitis that resulted in reproductive failures, whereas dogs similarly exposed to *Br. canis* did not. It was concluded that *Br. canis* would not cause abortions frequently unless bitches were exposed very early in gestation or before conception. Prepuberal dogs infected with *Br. suis* appeared to enter a recovery phase soon after exposure, whereas prepuberal dogs exposed to *Br. canis* developed progressive infection and lymphadenopathy.

Serum antibody titers were measured by agglutination, mercaptoethanol, complement fixation, and gel diffusion precipitation tests. Titers of *Br. canis*-infected dogs reached high levels and generally increased throughout the course of experiments. Titers of *Br. suis*-infected dogs increased rapidly until about 3 weeks postexposure and receded.

Bacteremia in *Br. canis*-infected dogs reached levels greater than 1×10^4 organisms/ml. of blood and persisted at greater than 1×10^3 organisms/ml. for at least 12 weeks in 2 dogs. In 95% of the samples from *Br. canis*-infected dogs,

bacteremia was detected by direct plating of blood. Conversely, the greatest magnitude of bacteremia detected in Br. suis-infected dogs was 80 organisms/ml., and most samples contained less than 2 organisms/ml. of blood. Mean numbers of organisms recovered from lymph nodes at necropsy provided a similar contrast. The mean Br. canis/Gm. was approximately 5×10^7, while the mean Br. suis/Gm. was approximately 1×10^5.

Pathologic changes in tissues were evaluated with the aid of gross observations, conventional histopathology, immunofluorescent techniques, and limited electron microscopy. Dogs infected with Br. suis usually developed pathologic alterations within 4 weeks after exposure. The predominant findings were diffuse metritis and placentitis, but lymphadenitis, focal mastitis, and focal hepatitis were also observed frequently. The most prominent placental changes were suppuration, necrosis, and accumulation of Br. suis at the junction of the spongy layer and placental labyrinth and it was postulated that this alteration was the basic cause of placental detachment and abortion. Nonviable fetuses and stillborn pups from Br. suis-infected bitches had only autolytic changes.

Pathologic changes in Br. canis-infected dogs seldom occurred in less than 6 weeks postexposure. The most frequent changes were lymphadenitis and focal hepatitis. The lymphadenitis was mainly characterized by lymphoid hyperplasia and plasmacytosis. A male dog exposed to Br. canis at 4 months of age developed lymphadenitis, epididymitis, prostatitis,

hepatitis, and nephritis during a 20-week postexposure period. There were no pathologic changes in fetuses and neonatal pups as a result of Br. canis infection.

Overall, Br. canis infection in dogs was characterized by a high multiplicity of organisms in vivo, but a prolonged incubation period before discernible pathologic alterations developed. Brucella suis infection had the opposite characteristics.

Cattle, sheep, and swine were exposed to Br. canis by the conjunctival route to determine their susceptibility to the organism. There was no bacteriologic evidence that any of 6 pregnant heifers developed infection, but a slight antibody response occurred. Six of 12 swine developed persistent, localized infection in lymph nodes of the head. However, there was no evidence that systemic infection developed in these pregnant gilts and sexually mature boars. Systemic, but transient, infection occurred in at least 7 of 12 sheep. Yearling rams, nonpregnant ewes, and pregnant ewes were equally susceptible. There were no observable clinical or gross pathologic effects of Br. canis infection in any of the above species. It was predicted that progressive, transmissible infection with Br. canis in domestic livestock would be a rare occurrence.

Brucella canis was of very low virulence for guinea pigs, compared to other Brucella. In addition, normal guinea pig peritoneal macrophages were far more capable of phagocytizing

and destroying Br. canis than Br. suis.

The characteristics of 41 different cultures of one strain of Br. canis were examined in detail. Brucella canis was distinguishable from other smooth and nonsmooth Brucella.

From the overall results, it was concluded that Br. canis is a discrete organism, causes a specific disease in dogs, and has a distinct host range.

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